ALKALOIDS OF CATHARANTHUS ROSEUS AND THEIR HYPOGLYCEMIC ACTIVITY

TIONG SOON HUAT

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

ALKALOIDS OF CATHARANTHUS ROSEUS AND THEIR HYPOGLYCEMIC ACTIVITY

TIONG SOON HUAT

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **TIONG SOON HUAT** I/C/Passport No: **860303-52-5817**

Registration/Matric No.: **SGR090147**

Name of Degree: **MASTER OF SCIENCE**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

"ALKALOIDS OF CATHARANTHUS ROSEUS AND THEIR HYPOGLYCEMIC ACTIVITY"

Field of Study**:** **NATURAL PRODUCT CHEMISTRY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work,
(2) This Work is original,
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work,
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work,
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained,
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

(Candidate Signature) Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name **PROFESSOR DR KHALIJAH AWANG**

Designation

ABSTRACT

Catharanthus roseus is a medicinal shrub plant used in traditional medicine treatment by local in India, South Africa, China and Malaysia for diabetes. Six known alkaloids and one new alkaloid namely, vindoline, vindolinine, perivine, vindorosine, vindolicine, serpentine and vindogentianine were isolated and identified from dichloromethane extract of *Catharanthus roseus'* leaves.

The leaves dichloromethane extract of *Catharanthus roseus* and alkaloids were not cytotoxic towards β-TC6 cells at 50.0 µg/mL except vindolinine and perivine with IC_{50} at 20.5 ± 3.6 and 46.7 ± 4.4 µg/mL**.** All five alkaloids tested showed higher glucose uptake in β-TC6 cells at treatment of 12.5 µg/mL and 25.0 µg/mL compared with untreated cells. Vindorosine, vindolicine and vindogentianine were shown to possess *in vitro* hypoglycemic activity for the first time. Vindolicine demonstrated highest hypoglycemic activity in glucose uptake and PTP-1B inhibition assay. The isolated alkaloid compounds could be responsible for the antidiabetic effect in *Catharanthus roseus* extracts and provide an explanation to its traditional usage.

ABSTRAK

Catharanthus roseus adalah satu tumbuhan herba ubatan yang digunakan oleh orang tempatan dalam perubatan tradisional di India, Afrika Selatan, China dan Malaysia untuk mengubati penyakit kencing manis. Enam alkaloid yang pernah dijumpai dan satu alkaloid baru: vindolin, vindolinin, perivin, vindorosin, vindolicin, serpentin dan vindogentianin telah berjaya diasingkan dan dikenal pasti daripada ekstrak dikhlorometane daun *Catharanthus roseus* (DE).

Hanya vindolinin dan perivin didapati membunuh cell β-TC6 pada kepekatan 50.0 µg/mL dengan IC_{50} 20.5 ± 3.6 and 46.7 ± 4.4 µg/mL. Kesemua lima alkaloid yang diuji menunjukkan meningkatan pengambilan gula dalam cell β-TC6 cells pada kepekatan 12.5 µg/mL and 25.0 µg/mL berbanding dengan cell yang tiada rawatan. Vindorosin, vindolicin and vindogentianin telah menunjuk activiti *in vitro* hypoglycemik untuk kali pertama. Vindolicin menunjukkan aktiviti hypoglycemik yang paling tinggi dalam penilaian pengambilan gula dan pembantutan PTP-1B. Alkaloid yang telah didapati daripada ekstrak daun *Catharanthus roseus* didapati bertanggungjawab ke atast aktiviti antidiabetik yang diperhatikan. Justeru, menyokong kebolehan tumbuhan ini dalam merawat penyakit kencing manis dalam perubatan traditional tempatan.

ACKNOWLEDGEMENT

I would like to express my special thanks and gratitude to my supervisor, Prof. Dr. Khalijah Awang who gave me the golden opportunity to do this challenging project on the topic **Alkaloids of *Catharanthus roseus* and their hypoglycemic activity,** which also provided support and guidance in enabling graduation from my master study. The assistances and advices offered by the late Assoc. Prof. Dr. Mat Ropi were also not forgotten.

I also wish to acknowledge the contribution made by Dr. Looi Chung Yeng, Mr. Mohamad Javad Paybar, Dr. Cheah Shiau Chuen and Dr. Aditya Arya in conducting the pharmacological studies covered in this project. The dry leaves of *Catharanthus roseus* were identified, collected and prepared by Mr. Teo, Mr. Din and Mr. Rafly. The help from Dr. Jalifah, Ms. Norzalida, Mdm. Suwing, Mr. Fateh and Mdm. Fiona for offering their NMR services were deeply appreciated. The facilitation supplied by the Department of Chemistry and Library of University of Malaya were remembered.

I wish to express my highest appreciation for the support from my friends and the members of phytochemistry laboratory. Last but not least, I would like to thank my family for all the love and encouragement. My parents always give me full support physically and mentally throughout my entire master study.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α	Alpha
β	Beta
δ	Delta
UV	Ultraviolet
mmu	Mili mass unit
ESI-TOFMS	Electron spray ionization-time of flight mass spectrometry
δ_H	Delta proton
δ_C	Delta carbon
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
dt	Doublet of triplets
m	Multiplet
br-d	Broad-doublet
br-s	Broad-singlet
br-q	Broad-quintet
s	Singlet
ε	Molar absorptivity
DEPT	Distortionless enhancement by polarization transfer
COSY	Correlation spectroscopy
HMQC	Heteronuclear multiple-quantum correlation spectroscopy
HMBC	Heteronuclear multiple-bond correlation spectroscopy
NMR	Nuclear magnetic resonance
H_a	Alpha (α) proton
H_b	Beta (β) proton
$M+H^+$	Molecular mass with proton adduct
OMe	Methoxy
Hz	Hertz
IR	Infared

OCOMe	Oxygenated acetyl (OAc)
t	Triplet
J	Coupling
q	Quintet
$[\propto]_D^{22}$	Specific rotation of alkaloid solution at 22°C under sodium D line
CHNS	Carbon, hydrogen, nitrogen, sulphur
$M+HCOO^-$	Molecular mass with formate adduct
ROS	Reactive oxygen species
PTP-1B	Protein-tyrosine phosphatase 1B
HE	Hexane crude extract of *Catharanthus roseus'* leaves
DE	Dichloromethane crude extract of *Catharanthus roseus'* leaves
ME	Methanol crude extract of *Catharanthus roseus'* leaves
WA	Water crude extract of *Catharanthus roseus'* leaves
IC_{50}	Half maximal inhibitory concentration
R^2	R squared (coefficient of determination)
RK-682	(*R*)-3-Hexadecanoyl-5-hydroxymethyltetronic acid
FT	Fourier transfer
TLC	Thin layer chromatography
EA	Ethyl acetate
TEA	Triethylamine
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
LCMS-IT	Liquid chromatography mass spectrometry-ion trap
PDA	Photodiode array
DAD	Diode array detection
PBS	Phosphate buffered saline
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fluorescence correlation spectroscopy
SEM	Standard error of the mean

CHAPTER 1

1.1 Introduction

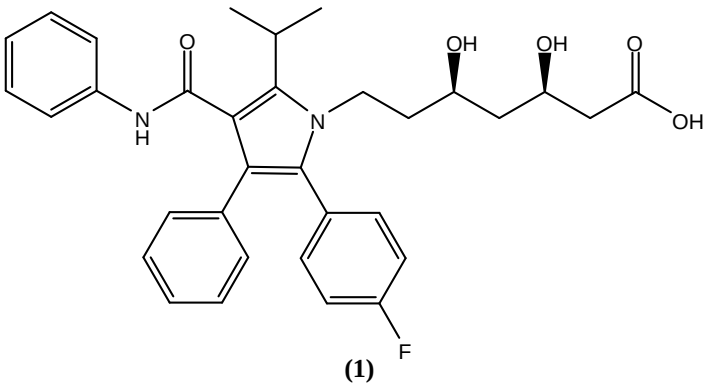
Nature has been providing food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours and fragrances and not least, medicines for humans throughout our civilization. The thousands years old traditional medicine system continues to provide mankind with new remedies[1] or insight for drug discovery.

Drug discovery from medicinal plants involve numerous scientific fields and various method of analysis. It begins with botanists, ethnobotanists, ethnopharmacologists or plant ecologists who collect and identify the plants. Natural product chemists will prepare extracts from the plant materials before subjecting these extracts to biological screening together with isolation and characterization of the active compounds through bioassay-guided fractionation. Molecular biologists will determine and implement appropriate screening assays directed toward physiologically relevant molecular targets.[2]

The natural products field had been and still productive. In the period of 01/1981-12/2010, there were only 29% of the new chemical entities that were approved as drug for treatment were synthetic in origin. Thus, reflecting the big contribution of natural product, other than synthetic on drug discovery and approval. The best selling drug of all is atorvastin **(1)**, a hypocholesterolemic descended directly from a natural product, which sold over $ 11 billion in 2004.[3]

Newman *et al.* (2012) reported that over half of drugs approved as antibacterial, antiviral, antiparasitic and anticancer were naturally derived products. However, there had been a decline in the research and development programs output of the pharmaceutical companies with a drop in the number of drugs launched during 2003 to 2010.[3] However, there were two approved drugs that mark the breakthrough of natural

products during that period, Prialt and Hemoxin. Prialt is a peptide toxin for pain relief from cone snail, a marine organism. It was the first approved pharmaceutical from the sea. Hemoxin is a mix of plants that came from native healer in Nigeria for treatment of sickle cell anemia. Therefore, Hemoxin can be regarded as a true ethnobotanical prepared medicine.[4]



(1)

However, there were only 37 approved antidiabetic drugs in the period of 30 years from 1981 to 2010. Out of the 37 drugs approved only 5 or 16.2% were from natural or inspired from natural, while a big proportion of 48.6% are peptide from biological.[3] Although antidiabetic drugs from nature were only a small contributor, there are still high potential in natural product, especially plants as the source of antidiabetic drugs. 45 medicinal plants in India showed varying degree of hypoglycemic and anti-hyperglycemic activity.[5] In traditional Chinese medical system, there were 86 natural (82 from plants and 4 from animals or insects) medicines were utilized in therapy of diabetes mellitus.[6]

Diabetes mellitus is a chronic metabolic disorder that results from a failure of the body to produce the hormone insulin and/or inability of the body to respond adequately for insulin circulation.[7] There are 2 major types out of 5 classification introduced by American Diabetes Association; type 1 and type 2 diabetes. Type 1 diabetes is also known as insulin dependent diabetes because the patient loses the ability

to secrete insulin due to selective autoimmune destruction of pancreatic β-cells.[7-9] The most common type 2 diabetes is also known as non-insulin dependent diabetes because the body does not respond well to insulin and the normal insulin level produce by pancreas aren't sufficient. Type 2 diabetes is becoming more common due to increasing obesity and failure to exercise especially win urban lifestyles.[8]

Diabetes has become the third most common disease that heavily threatens human health around the world, following cardiovascular diseases and cancers. The latest data from the World Health Organization (WHO) approximates there are 346 million people worldwide are living with diabetes today. It is estimated that the number of people with diabetes will double by 2030. To date, no cure had been identified.[7; 10]

In Malaysia, the number of diabetic has increased by almost 80% in the last 10 years from 1996-2006 to 1.4 million adults above the age of 30. This number had increase about two fold to 3 million now. The Malaysian National Health Morbidity Survey III (NHMS III) conducted in 2006 show that our national prevalence of diabetes was 14.9% which we observed about 80% rise from 8.3% in NHMS II. Out of the majority patients (70%) that were on oral medication, only 7.2% were on insulin alone or in combination. Meanwhile, only 0.6% took traditional medicine for diabetes. However, patients were known to combine prescribed medications with alternative treatment including the use of local herbs in real clinical practice.[11]

The use of herbal medicines for treatment of diabetes mellitus has gain attention throughout the world. WHO also had recommended and encouraged this practise in 1980. Although oral hypoglycaemic agents/insulin are the mainstay of the treatment but it have prominent side effects and fail significantly in altering the course of diabetes' complication. Therefore, many people are turning to complementary therapies which are

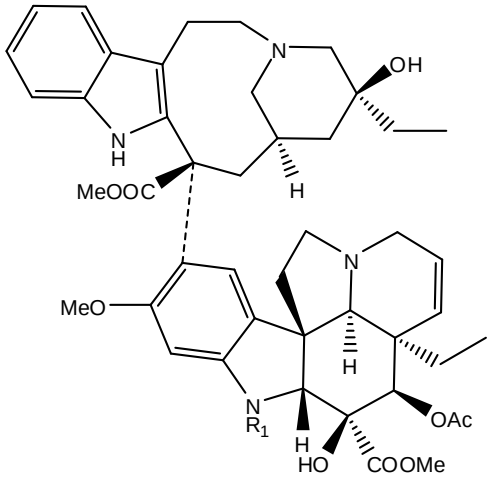
medicinal plants arising from indigenous medicines such as *Catharanthus roseus* that was widely used for the treatment of diabetes especially in Asia and Africa.

1.2 *Catharanthus roseus* the antidiabetic medicinal plant

Catharanthus roseus (L.) G. Don was a renowned medicinal plant from the family of Apocynaceae.[12] It was also known as Madagascar Periwinkle and Linnaeus named it as *Vinca rosea* in the 10th edition of his Systema Naturae in 1759. In 1837, G. Don observed many botanical differences between *Vinca* and the Madagascar Periwinkle which he later moved to a separate genus *Catharanthus*.[13]

Catharanthus roseus was indigenous to Madagascar. It has been widely cultivated as ornamental[13] for hundreds of years and can be found growing wild in most warm regions of the world.[12] The plant was commonly grown in gardens for beddings, borders and for mass effect. It blooms throughout the year and is propagated by seeds or cuttings.[12] In the more temperate regions, the plant can only be used as an indoor annual potplant because it will die at first frost during winter if placed outdoor. Otherwise, a conservatory or greenhouse was usually recommended to keep the plant alive and flowering all year long in temperate regions.[12]

The leaves of *Catharanthus roseus* were used in traditional medicine as an oral hypoglycemic agent and the study of this activity led to the discovery of two terpenoid indole alkaloids, vinblastine **(2)** and vincristine **(3)**, the first natural anticancer agents to be clinically used. Since the discovery of these two bisindole alkaloids from *Catharanthus roseus*, studies were concentrated onto the anticancer activity and less into the antidiabetes activity from this plant.



(2), R_1=CH_3
(3), R_1=CHO

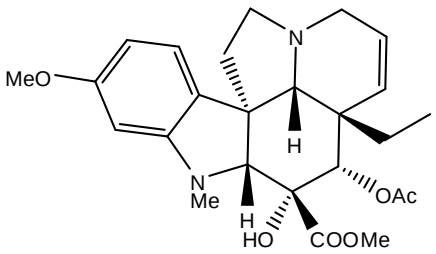
Herbal diabetic treatments using *Catharanthus roseus* were popularly used because it has high therapeutic effect with minimum side effects and were cost effective. *Catharanthus roseus* was widely used in the treatment of diabetes especially in India[14] and South Africa[15]. In Malaysia, the decoction of *Catharanthus roseus* was used to treat diabetes, reduce blood pressure, insomnia and cancer.[16] The local native tribe of Temuan in Malaysia had been reported to cultivate *Catharanthus roseus* as a medicinal plant.[17]

The twig and leaves of *Catharanthus roseus* showed significant increase of glucose utilisation in organic and aqueous extract.[15] The sap of fresh leaves had reduced blood glucose in alloxan-treated rabbits.[18] Another study showed that dichloromethane:methanol (1:1) extract of flowering twigs possess antidiabetic activity in streptozotocin-induced diabetic rat. There was significant increase in glucokinase activity in the rats' liver treated. Thus, suggesting there were an increase in utilization of glucose as the mechanism.[19]

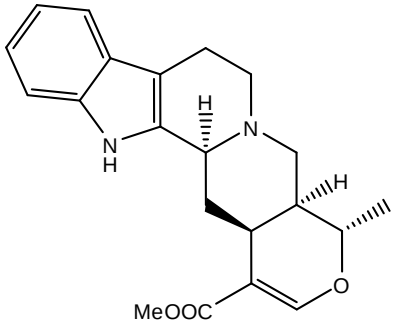
The hypoglycemic activity from *Catharanthus roseus* was due to the presence of phytochemical such as vindoline **(4)**, tetrahydroalstonine **(5)**, catharanthine **(6)**,

lochnericine **(7)**, leurosine **(8)**, vindolinine **(9)**, adenosine **(10)**, β-sitosterol **(11)**, quercetin **(12)**, ursolic acid **(13)** and tannic **(14)**.[20-22]

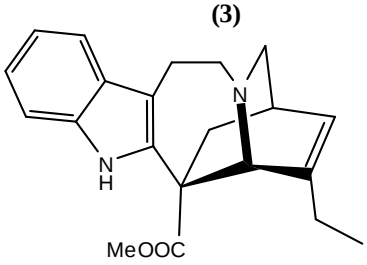
Recent researches on the antidiabetic activity of *Catharanthus roseus* was orientated toward producing the right herbal medicine for treatment of diabetes by evaluation of diabetes complication and side effect of diabetes drug with introduction *Catharanthus roseus* extract.[23] Researchers were interested to study the synergistic effect of drug and plant extract by co-administration.[24] Another study had been conducted to determine the safety of gliclazide **(15)**, an anti-diabetic drug usage together with aqueous extract of *Catharanthus roseus*.[21]



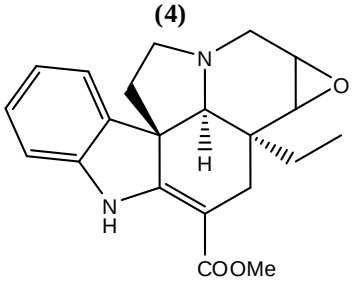
(3)



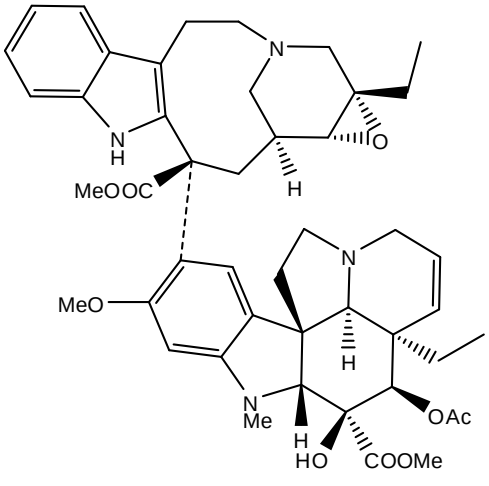
(4)



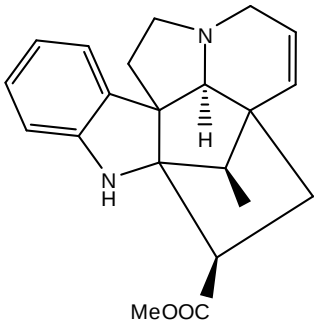
(5)



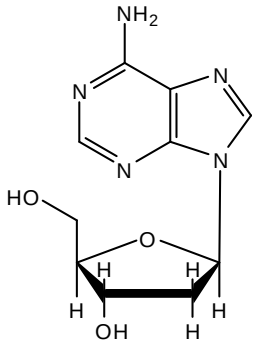
(6)



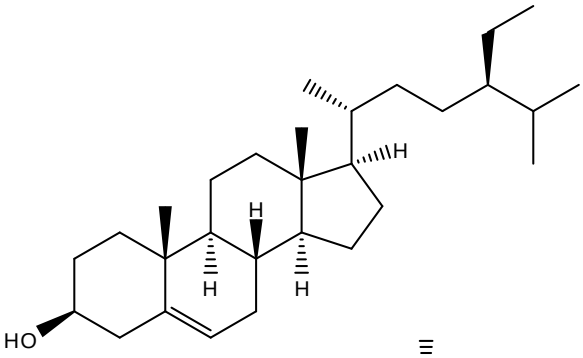
(7)



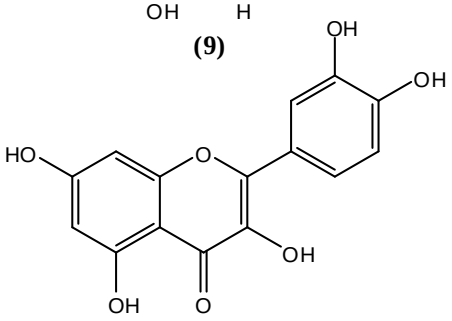
(8)



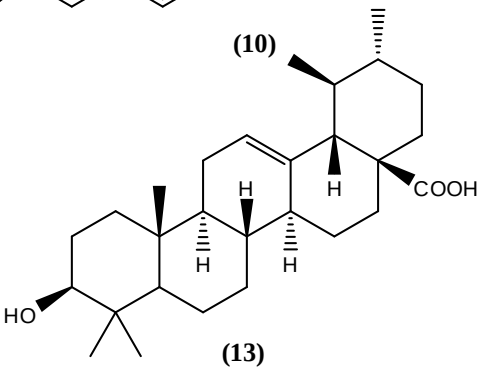
(9)



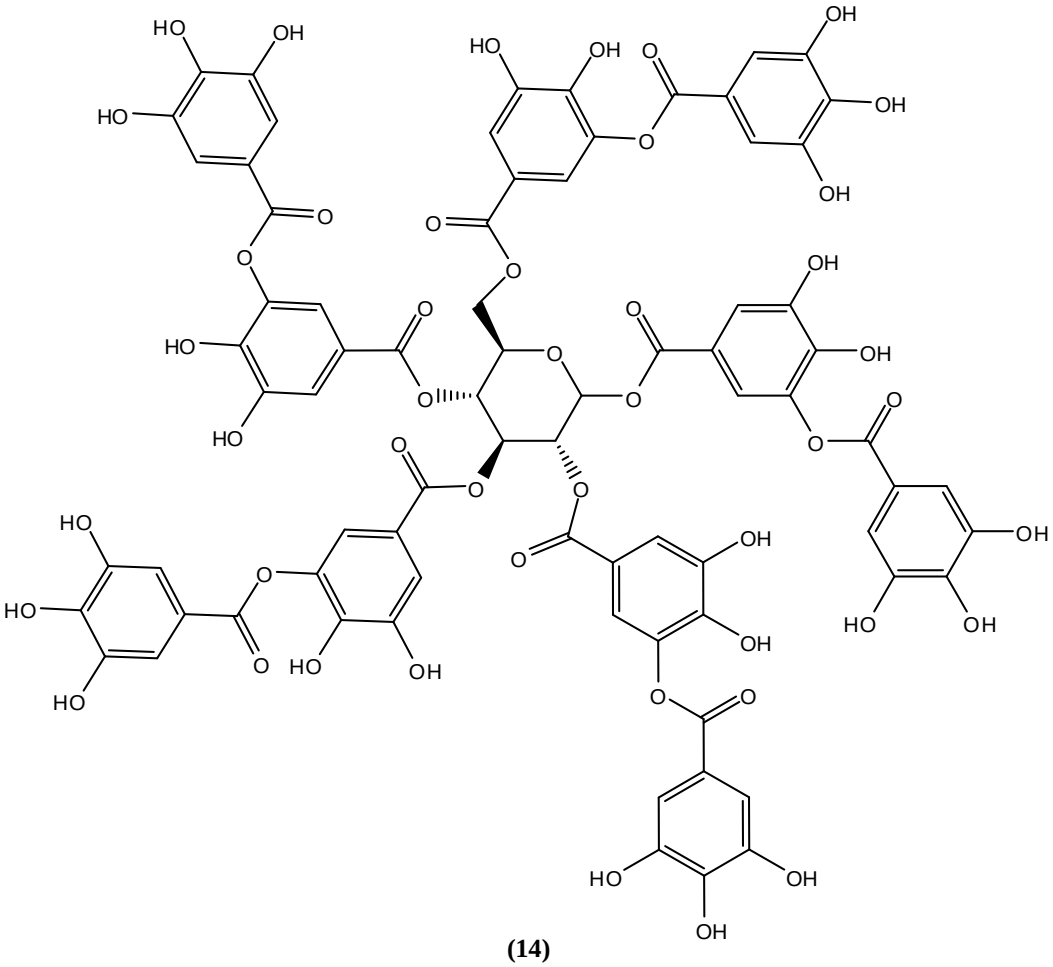
(10)



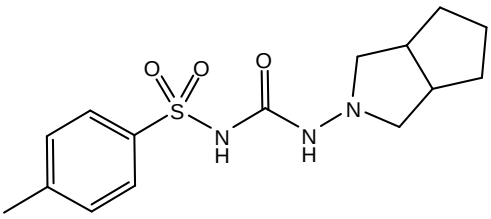
(12)



(13)



(14)



(15)

1.3 Apocynaceae

This family name was given by A.L. de Jussieu in 1789.[13] Apocynaceae can also be known as periwinkle family which it consist of 180 genera and 1500 species. It is of mostly tropical distribution with a few in temperate regions.[25]

Apocynaceae species are usually twining shrubs and rarely erect with latex. Their leaves are simple of opposite or alternate or in whorls of 3 with close parallel lateral veins. They have panicle, cyme or raceme inflorescence or solitary flower with presence of bracts and bracteoles.[25]

Some plants from this family had been used as economic and ornamental plants. *Carissa macrocarpa* (*C. grandiflora*), Natal plum and *C. carandas* are grown for their edible fruit. *Hancornia speciosa*, Mangabeira also has edible fruit but in addition the sap is a source of rubber in South America. Silk Rubber is obtained from *Funtumia elastica*. The seeds of various species of *Strophanthus* have long been used for arrow poisons in Africa and some have been accepted as useful drugs in Western medicine. *Nerium oleander*, Oleander and *Plumeria rubra*, Frangipani have been used as decorative plants for warm regions or glasshouse cultivation in cooler climate.[25]

This family is divided into 2 subfamilies and 5 tribes as shown in Table 1.2 below.

Table 1.1: Classification of the plants in the family of Apocynaceae.[25]

Family	Subfamily	Tribe	Subtribe
Apocynaceae	**Plumerioideae**	Arduineae	Landolphia
			Carissa
			Allemanda
		Pleiocarpeae	Pleiocarpa
		Plumerieae	Rauwolfia
			Tabernaemontana
			Alyxia
			Aspidosperma
			Ochrosia
			Amsonia
			Plumeria
			Vinca
			Catharanthinae
	Apocyniodeae	Apocyneae	Mandevilla
			Strophanthus
			Dipladenia
			Apocynum
			Nerium
		Parsonsieae	Parsonsia
			Prestonia
			Forsteronia

1.4 *Catharanthus* G.Don

The *Catharanthus* genus belongs to the subtribe Catharanthinae in the tribe Plumerieae, subfamily Plumeroideae within the family of Apocynaceae. This genus comprises of eight species namely *Catharanthus roseus, Catharanthus trichophyllus, Catharanthus lanceus, Catharanthus ovalis, Catharanthus longifolius, Catharanthus scitulus, Catharanthus coriaceus* and *Catharanthus pusillus*.[13]

Catharanthus are annual or perennial herbs or undershrubs which are often with white latex and woody at the base. It has herbaceous to fleshy-coriaceous leaves with terminal or axillary inflorescences. It has 5-merous and actinomorphic flowers with narrowly to narrowly triangular sepals. It has salver-form corolla that come in purple, red, pink or white colour.[13]

Out of the eight species, seven are endemic to Madagascar and one (*Catharanthus pusillus*) is endemic to India. Several species grow more or less in the same area within Madagascar but their core area can be distinguished. *Catharanthus trichophyllus* can be found mostly in the north-western part of Madagascar. Within the central part of Madagascar, *Catharanthus lanceus* core area is around Antananarivo, *Catharanthus coriaceus* around Itremo Mts., *Catharanthus ovalis* around Parc Isalo and *Catharanthus longifolius* around Ambalavao. *Catharanthus scitulus* can be found in south-central part of Madagascar. *Catharanthus roseus* is thought to originate from Fort Dauphin area at the most south-eastern part of Madagascar. However, *Catharanthus roseus* has been cultivated as ornamental all over the tropics and subtropic.[13] As a result, it is now naturalized in many countries and escaped in many areas of Madagascar.[25]

In 1753, Linnaeus described the genus *Vinca* with two species namely *Vinca major* and *Vinca minor*. He added *Vinca rosea* to this group four years later. Reichenbach separated *Vinca rosea* from other species in the genus *Vinca* and giving *Lochnera* as the generic name for the species in 1828. Endlicher made a clear distinction between the genus *Vinca* and *Lochnera* in his Genera Plantarum which was published in August 1838. However, George Don had published the first part of his General System of Gardening and Botany volume IV which he also made a separation between the species in genus *Vinca* and giving the name *Catharanthus* as the new genus in 1837. Therefore, *Catharanthus* got priority over *Lochnera* which was published one year later.[13]

1.5 *Catharanthus roseus*

Catharanthus roseus is an undershrub plant that grows to 30-100 cm high, either erect or decumbent with white latex and come with unpleasant smell. Its trunk can grow

up to 1 cm in diameter and pale grey in colour. It has terete, narrowly winged, green or dark red, pilose or glabrous stem. It has decussate and petiolate leaves. Its flowers are axillary, solitary or paired, pendunculate 1-4 mm long, narrowly winged and glabrous or pilose. It has medium green sepals and pink, magenta or white with darker center, paler or whitish outside corolla lobes. The fruits are green or pale green and seeds are black.[13]

Catharanthus roseus are found at the altitude of 0-900 m on coral sand, beaches and limestone rocks. It can also survive in open forests, ruderal places, along roadsides in dry shrub woodland or grassland. As the result of the high survivability in wide variety of habitat and flowering throughout the year, it has been naturalized and cultivated as indoor or garden plants all over the tropic and subtropics.[2]

In Malaysia, *Catharanthus roseus* has also been cultivated and naturalized throughout the country. This plant is known by the locals with a few names such as Tahi Ayam, Kemuning Cina,[26] Kembang Sari Cina, Kemunting Cina, Rumput Jalang and Tapak Dara.[27; 28]

Cultivation of this plant has been going on for a very long time. Even before it was studied for its medicinal value, it was cultivated as a garden or decorative plant. The seed of this plant was first sent to the Royal Gardens in Versailles near Paris from Madagascar.[13]

In 1661, the hypotensive effect of the root extract was reported. Around 1930, pharmacologists were attracted to study its antidiabetic activity due to its traditional use against diabetes. This experiment was repeated at the 1950's, but the test animals became seriously ill. As the result, Noble and co-workers discovered vinblastine **(2)** as an unexpected myelosuppressive agent in 1958 during their search for an antidiabetic agent in *Catharanthus roseus*.[29]

Independently, researchers at Eli Lily found extracts of *Catharanthus roseus* possessed activity against P-1534 leukaemia in mice and isolated vinblastine **(2)** as its active entity in 1959. The structure of a related compound, vincristine methiodide was then determined by an x-ray crystallography in 1965.[30] Vinblastine **(2)** and vincristine **(3)** were the first natural anticancer agents to be clinically used.[31]

1.6 Objectives of the study

In continuing interest to investigate Malaysian medicinal plants, the author has embarked on a study of the antidiabetic agents from *Catharanthus roseus*. The objectives of this study are as follows:

I. To isolate the alkaloids constituents in the leaves of Malaysian *Catharanthus rosues*.

II. To identify the alkaloids constituents isolated from the leaves of Malaysian *Catharanthus roseus* by spectroscopic methods.

III. To evaluate the cytotoxicity of the alkaloids isolated against normal pancreas cells (β-TC6)

IV. To evaluate the antidiabetic activity of the alkaloids by glucose uptake in β-TC6 cells while studying it possible mechanism by PTP-1B inhibition of some most promising alkaloids as antidiabetic agent.

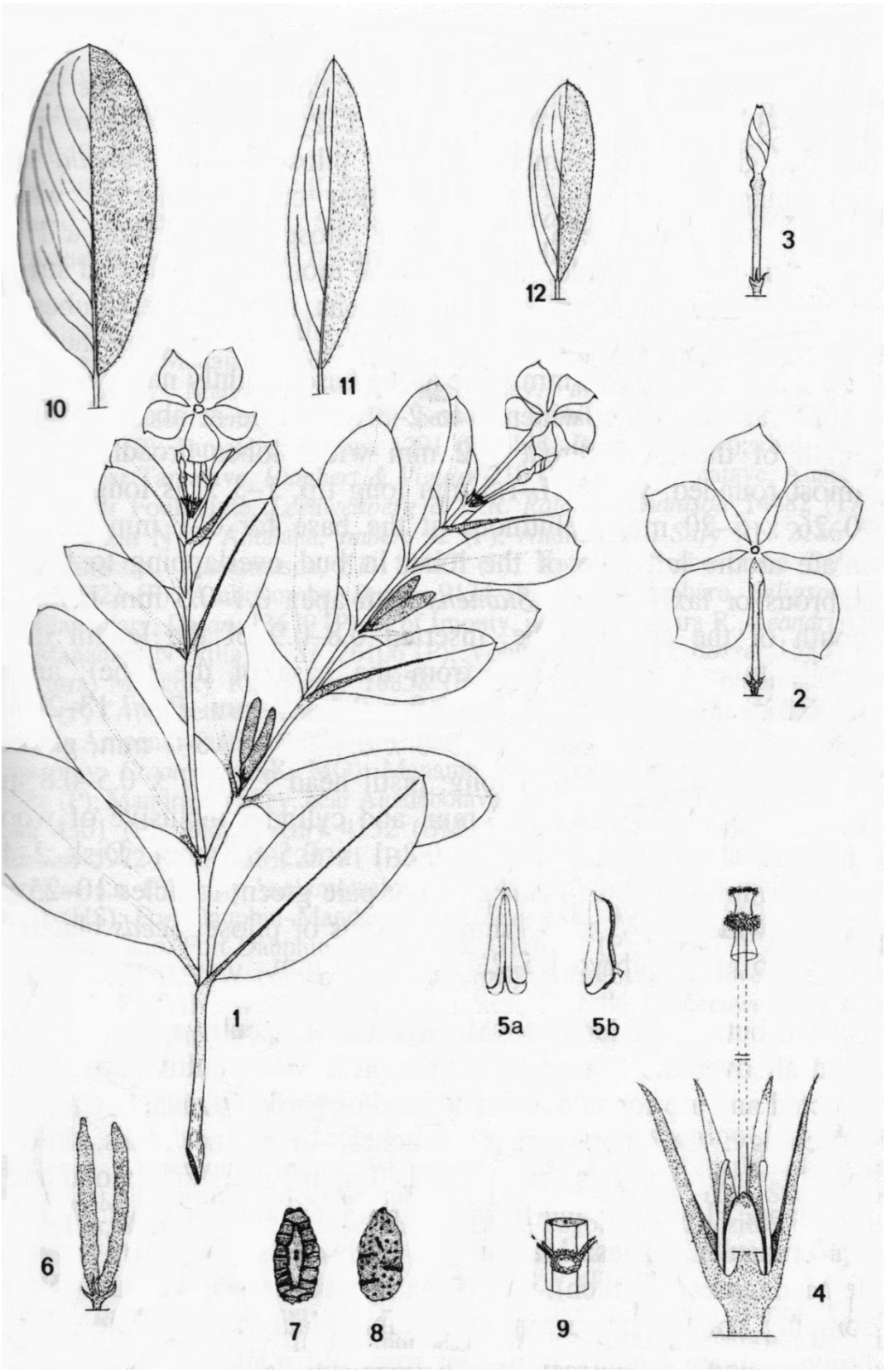


Figure 1.1: *Catharanthus roseus*. 1, habit (×2/3); 2, flower (×2/3); 3, bud (×2/3); 4, pistil (×6 2/3); 5, anthers (×6 2/3); 6, fruit (×2/3); 7, seed, back side (×6 2/3); 8, seed, hillar side (×6 2/3); 9, detail stem (×3 2/3); 10, 11, 12, leaves (×2/3).[13]

CHAPTER 2

2.1 Chemical constituents of *Catharanthus roseus*

Catharanthus roseus is a well known Apocynaceae medicinal plant, rich with indole alkaloids. However, this plant also consists of other valuable constituents in addition to indole alkaloids such as phenolics, terpene and others.

R.Verpoorte *et al.* reviewed the occurrence of phenolic compounds such as 2,3-dihydrobenzoic acid, phenylpropanoids, flavonoids and athnocyanins in *Catharanthus roseus*.[32] Ferreres *et al.* conducted a study on noncoloured phenolics in *Catharanthus roseus*, which allowed characterization of three caffeoylquinic acids and fifteen new flavonol glycosides.[31] K.Toki *et al.* and I.M.Chung *et al.* research had showed more new and interesting phenolic compounds from *Catharanthus roseus*.[33; 34] P.Guedes De Pinho *et al.* had identified a total of 88 volatile and semi-volatile component which including diterpenic compounds, sesquiterpenes and some pyridine, pyrazine, indole and carotenoid derivatives.[35]

Indole alkaloids are commonly found in plants from the family of Apocynaceae, Loganiaceae and Rubiaceae as monoterpene indole alkaloids. Plants from the families of Leguminoseae, Rutaceae, Simaroubaceae, Zygophyllaceae, Elaeocarpaceae and Alangiaceae are also known to have indole alkaloids but are less studied. In Apocynaceae, indole alkaloids are known to be present in 32 genera such as *Hunteria*, *Kopsia*, *Alstonia*, etc. These indole alkaloid-bearing genera of Apocynaceae come from the same subfamily of Plumeriodeae and are represented by 4 tribes, Carisseae, Tabernaemontaneae, Rouvolfieae and Plumerieae (Table 2.1).[36]

Leeuwenberg in 1980 had categorized monoterpene indole alkaloids into eight main skeletal types: corynanthean, strychnan, ibogan, plumeran, eburnan, aspidospermatan, vallensiachotaman and vincosan. (Figure 2.1) Beside these eight types of indole alkaloids, there were other unknown and miscellaneous types that were not

covered by Leeuwenberg. Eventhough there were other catergorization of indole alkaloids were reported, the author found the classification by Leeuwenberg was less complicated and have clear relationship showed in between each skeleton of indole alkaloids (Figure 2.1).[36]

Table 2.1: Genera of Apocynaceae which have species containing indole alkaloids.[36]

Family	Subfamily	Tribe	Subtribe	Genera
Apocynaceae	Plumerioideae	Carisseae	Carissinae	Melodinus Leuconotis
			Landolphiinae	Landolphia
			Pleiocarpinae	Picralina Hunteria Pleiocarpa
		Tabernaemontaneae	-	Crioceras Callichilia Stemmadenia Capuronetta Tabernaemontana Tabernathe Voacanga Schizoxygia
		Rouvolfieae	Rauvolfiinae	Cabucala Rauvolfia
			Ochrosiinae	Ochrosia
			Vallesiinae	Vallesia Kopsia
			Condylocarpinae	Condylocarpon
		Plumerieae (Alstonieae)	Craspidosperminae	Craspidospermum
			Plectaneiinae	Gonioma
			Alstoniinae	Alstonia Tonduzia
			Aspidospermatinae	Diplorhynchus Aspidosperma Geissosperum
			Catharanthinae	Rhazya Amsonia Catharanthus Vinca Haplophyton

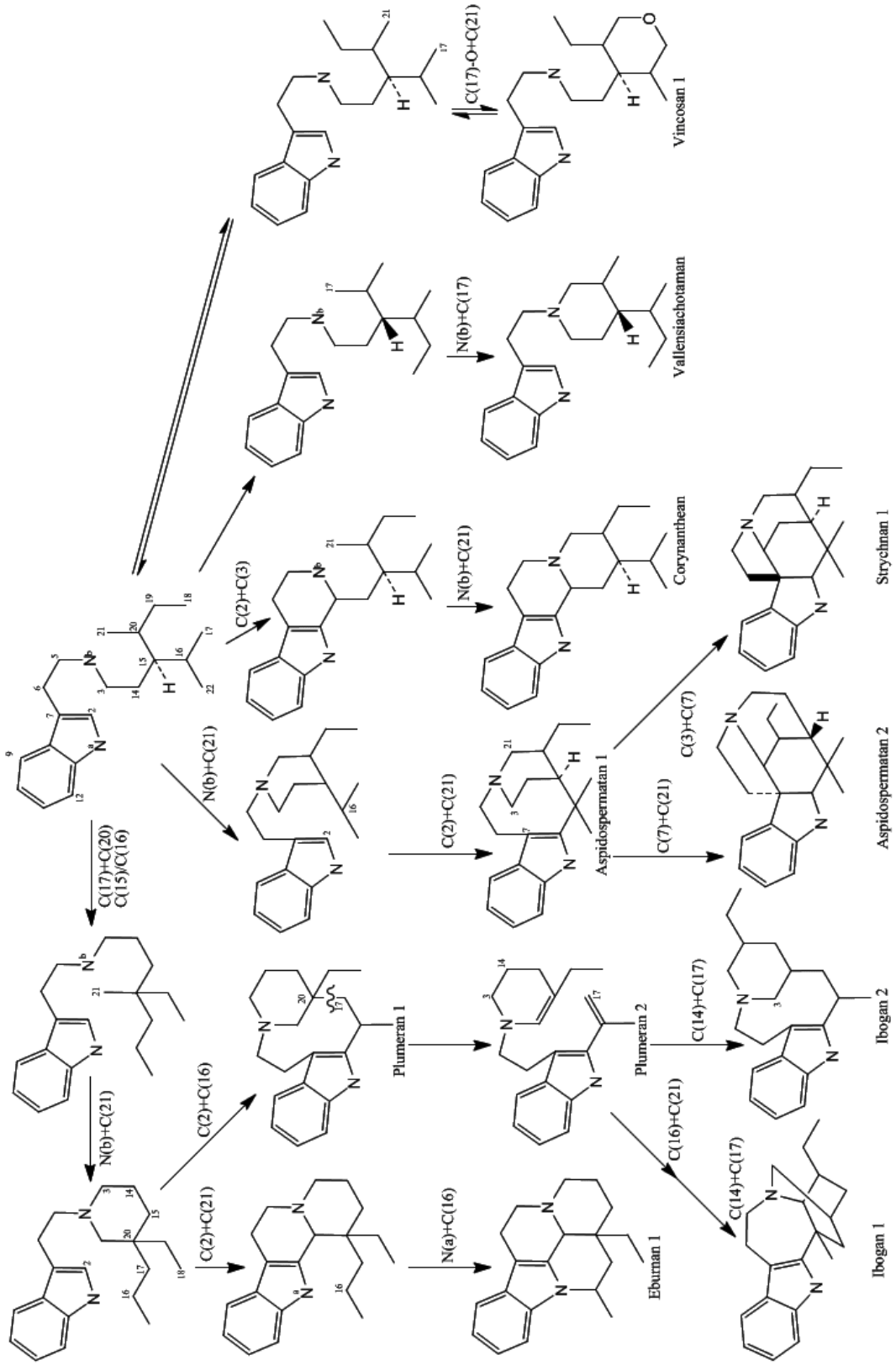


Figure 2.1: The biogenetic relationships of the 8 main skeletal types.[38]

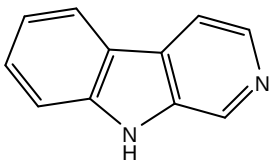
Catharanthus roseus as a species from the tribe of Plumerieae and subtribe of Catharanthine has more than 130 indole alkaloids with more than 25 being dimeric bisindoles. Bisindoles from *Catharanthus roseu*s found to be derived from monomers of one or two main skeletal types but mostly are from plumeran and ibogan type. Even after thorough and comprehensive investigations of the chemical constituents from this plant, recently Wang *et al.* (2011) still able to report three new indole alkaloids from this plant.[37] Table 2.2 list down all the indole alkaloids that had been reported from *Catharanthus roseus*.

Table 2.2: Known alkaloids according to biogenetic class isolated from *Catharanthus roseus*.

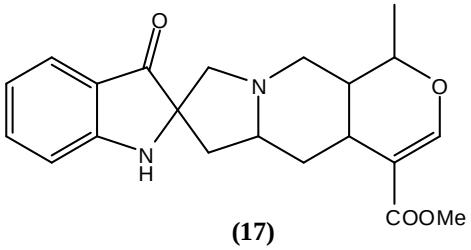
Alkaloids	Class	Alkaloids	Class
Vinblastine **(2)**[38]	B	Vincristine **(3)**[39]	B
Vindoline **(4)**[38]	P	Tetrahydroalstonine **(5)**[40]	C
Catharanthine **(6)**[40]	I	Lochnericine **(7)**[41]	P
Leurosine **(8)**[42]	B	Vindolinine **(9)**[41]	P
β-carboline **(16)**[43]	M	Pseudoindoxylajmalicine **(17)**[44]	M
Apparicine **(18)**[45]	M	N-oxidefluorocarpamine **(19)**[46]	M
Mitraphylline **(20)**[38]	M	Rosamine **(21)**[47]	M
N,N-dimethyltryptamine **(22)**[41]	M	N_b-acetyltryptamine **(23)**[40]	M
Akuammicine **(24)**[42]	S	12-hydroxyakuammicine **(25)**[48]	S
Lochneridine **(26)**[38]	S	Alioline **(27)**[49]	I
Coronaridine **(28)**[50]	I	3-epiajmalicine, **(29)**[44]	C
Akuammigine **(30)**[40]	C	Hydroxyindolenineajmalicine, **(31)**[44]	C
O-deacetylakuammiline, **(32)**[51]	C	10-hydroxyldeacetylakuammiline **(33)**[44]	C
Strictamine **(34)**[52]	C	18-hydroxystrictamine, **(35)**[52]	C
Akuammine **(36)**[53]	C	Alstonine **(37)**[40]	C
Anthirine **(38)**[54]	C	21-hydroxycyclolochnerine, **(39)**[55]	C
Lochnerine **(40)**[56]	C	Pericyclivine **(41)**[42]	C
N-oxidelochnerine **(42)**[37]	C	11-methoxy-N-oxide-lochnerine **(43)**[37]	C
N-oxidenormacusine B **(44)**[37]	C	Perivine **(45)**[38]	C
N_4-formylperivine, **(46)**[42]	C	Pleiocarpamine **(47)**[54]	C
Sitsirikine **(48)**[40]	C	Dihydrositsirikine **(49)**[54]	C
19,20-*cis*-16-(*R*)-isositsirikine **(50)**[55]	C	19,20-*trans*-16-(*R*)-isositsirikine **(51)**[55]	C
19,20-*trans*-16-(*S*)-isositsirikine **(52)**	C	Yohimbine **(53)**[41]	C
Bannucine **(54)**[57]	P	Cathovaline **(55)**[58]	P
19-(S)-epimisilinec **(56)**[59]	P	Lochnerinine **(57)**[38]	P

Rosicine **(58)**[60]	P	Minovincinine **(59)**[61]	P
Vincadifformine **(60)**[62]	P	19-hydroxytabersonine **(61)**[60]	P
Venalstonine **(62)**[63]	P	Vincoline **(63)**[64]	P
Vindolicine **(64)**[37]	P	Vindorosine **(65)**[50]	P
19-epivindolinine **(66)**[65]	P	N_b-oxidevindolinine **(67)**[61]	P
19-epi-N-oxidevindolinine **(68)**[61]	P	Catharanthamine **(69)**[66]	B
Catharine **(70)**[67]	B	17-deacetoxyleurosine **(71)**[68]	B
Leurosinone **(72)**[69]	B	5'-oxoleurosine **(73)**[70]	B
21'-oxo-leurosine **(74)**[70]	B	N_b'-oxide-Leurosine **(75)**[71]	B
4-deacetoxyvinblastine **(76)**[68]	B	N_b-oxide-leurosidine **(77)**[72]	B
Roseadine **(78)**[71]	B	Roseamine **(79)**[71]	B
Pseudovinblastinediol **(80)**[73]	B	Deacetylvinblastine **(81)**	B
N-demethylvinblastine **(82)**	B	20-deoxyvinblastine **(83)**[74]	B
14'-hydroxyvinblastine **(84)**[73]	B	15'-hydroxyvinblastine **(85)**[75]	B
Vindesine **(86)**[76]	B	Vinamidine **(87)**[73]	B
3',4'-anhydrovinblastine **(88)**[77]	B	Vincathicine **(89)**[78]	B
Vindolicine **(90)**[67]	B	Vingramine **(91)**[79]	B
Methylvingramine **(92)**[79]	B	Strictosidine lactam **(93)**[41]	D
Vincoside **(94)**[80]	D	N-acetylvincoside **(95)**[80]	D
Tubotaiwine **(96)**[81]	A	Vincamine **(97)**[82]	E
Vallesiachotamine **(98)**[41]	V	Isovallesiachotamine **(99)**[81]	V
Vincarodine **(100)**[67]	E	Cathenamine **(101)**[83]	C
Ajmalicine **(102)**[41]	C	19-epiajmalicine **(103)**[54]	C
Serpentine **(104)**[40]	C	Tabersonine **(105)**[40]	P
11-methoxytabersonine **(106)**[67]	P	Deacetoxyvindoline **(107)**[84]	P
Deacetylvindoline **(108)**[67]	P	Preakuamicine **(109)**[85]	S

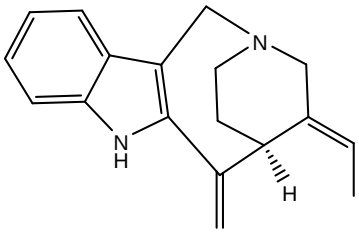
Class: A = aspidospermatan, B = bisindole, C = corynanthean, D = vincosan, E = eburnan, I = ibogan, M = miscellaneous, P = plumeran, S = strychnan, V = vallesiachotaman.



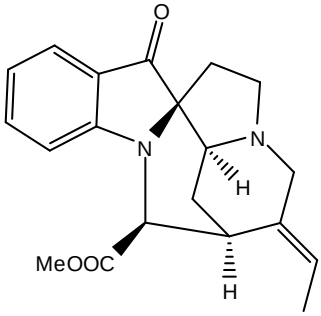
(16)



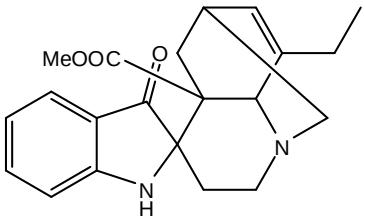
(17)



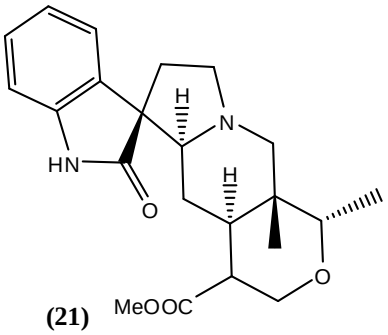
(18)



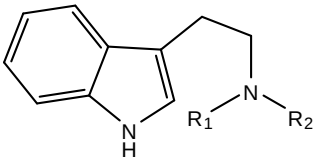
(19)



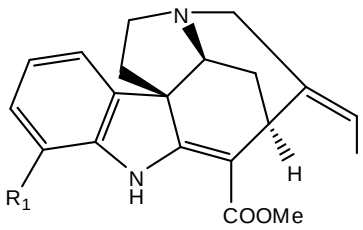
(20)



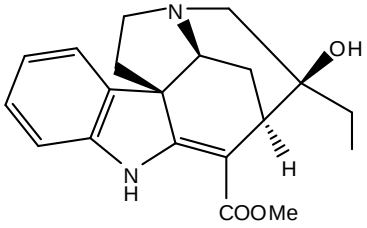
(21)



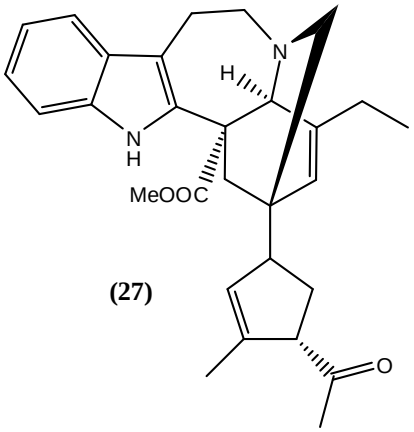
(22) R_1=CH_3, R_2=CH_3
(23) R_1=H, R_2=$COCH_3$



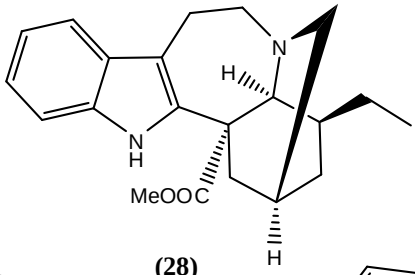
(24) R_1=H
(25) R_1=OH



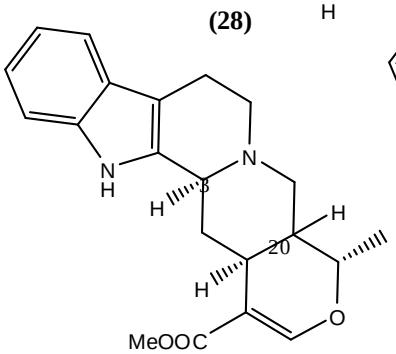
(26)



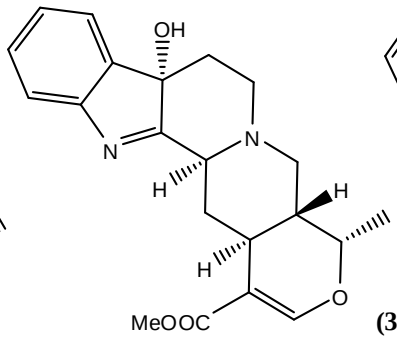
(27)



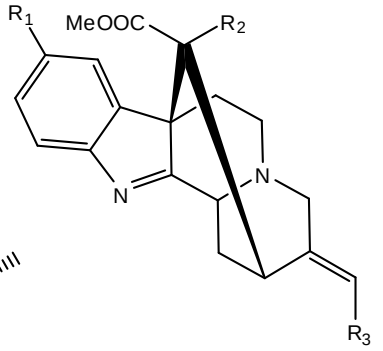
(28)



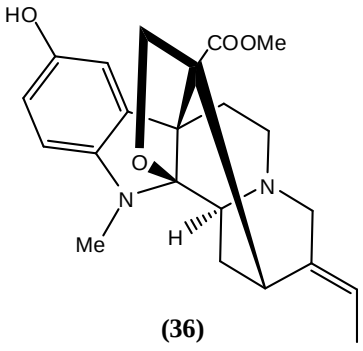
(29) C_{20}=*R*
(30) C_{20}=*S*



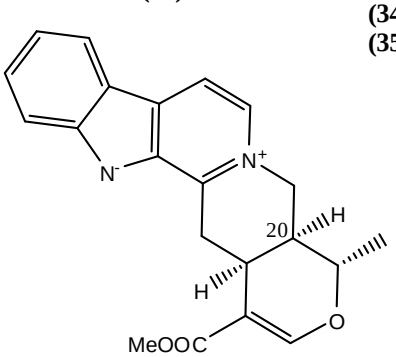
(31)



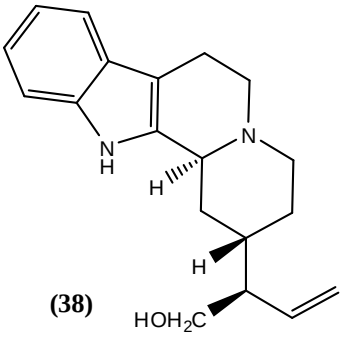
(32) R_1=H, R_2=CH_2OH, R_3=CH_3
(33) R_1=OH, R_2=CH_2OH, R_3=CH_3
(34) R_1=H, R_2=H, R_3=CH_3
(35) R_1=H, R_2=H, R_3=CH_2OH



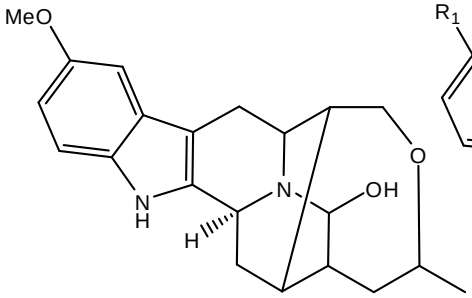
(36)



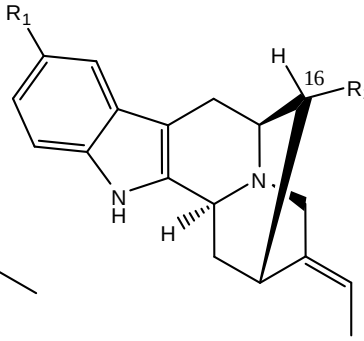
(37)



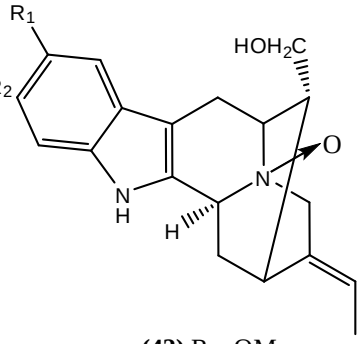
(38)



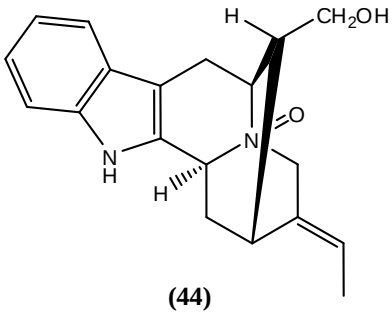
(39)



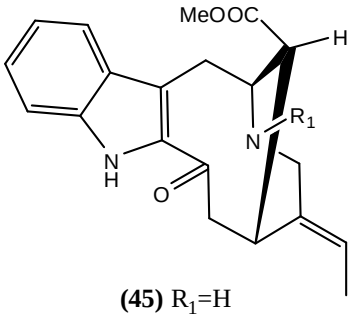
(40) C_{16}=*R*, R_1=OMe, R_2=CH_2OH
(41) C_{16}=*S*, R_1=H, R_2=COOMe



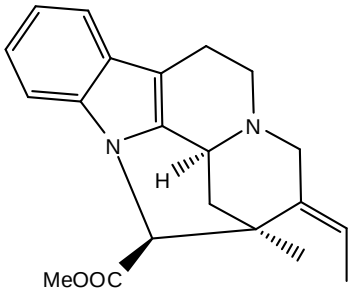
(42) R_1=OMe
(43) R_1=H



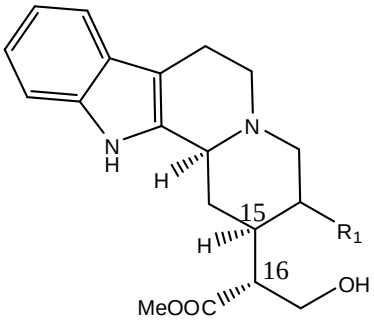
(44)



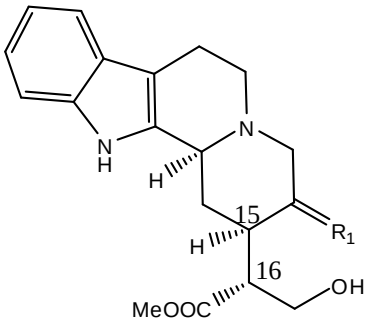
(45) R_1=H
(46) R_1=CHO



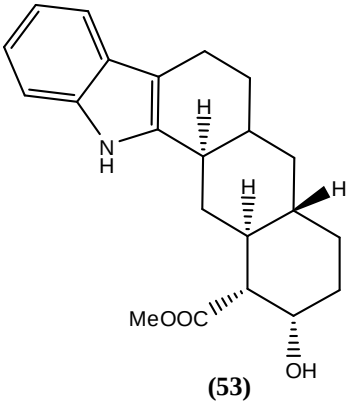
(47)



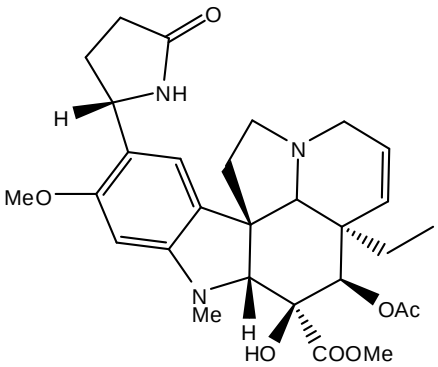
(48) R_1=$CHCH_2$
(49) R_1=CH_2CH_3



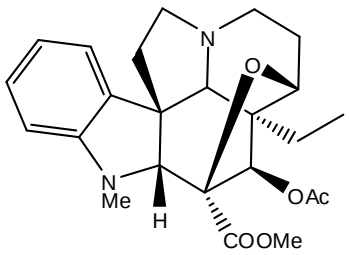
(50) R_1=*cis*$CHCH_3$, C_{16}=*R*
(51) R_1=*trans*$CHCH_3$, C_{16}=*R*
(52) R_1=*trans*$CHCH_3$, C_{16}=*S*



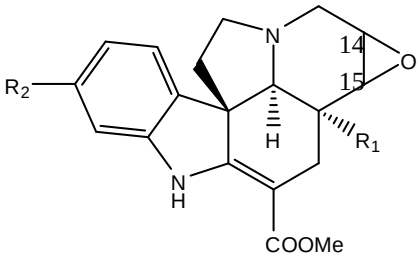
(53)



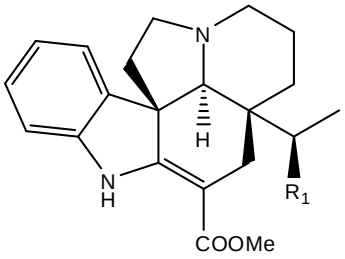
(54)



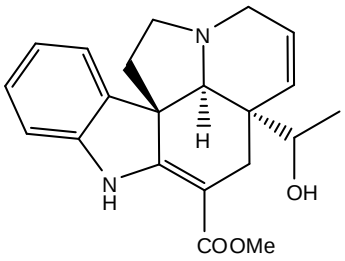
(55)



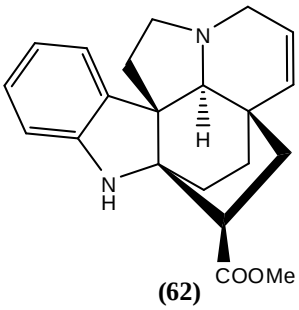
(56) R_1=(*S*)-$CHOHCH_3$, R_2=H
(57) R_1=CH_2CH_3, R_2=OMe
(58) R_1=H, R_2=H



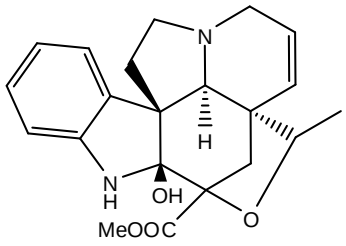
(59) R_1=OH
(60) R_1=H



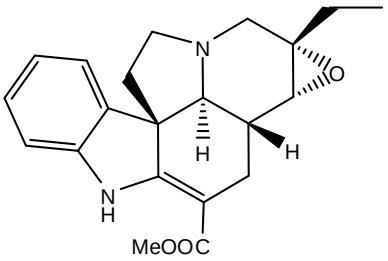
(61)



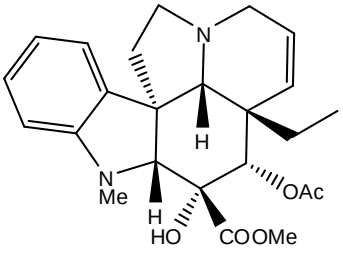
(62)



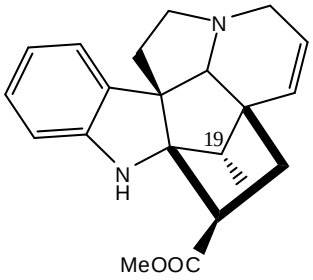
(63)



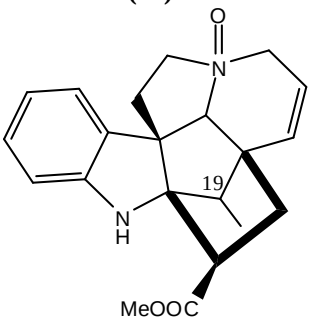
(64)



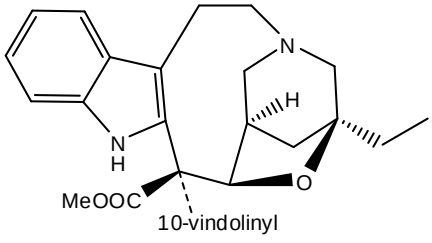
(65)



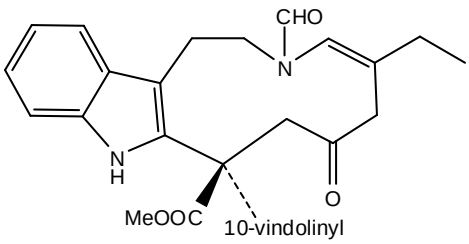
(66)



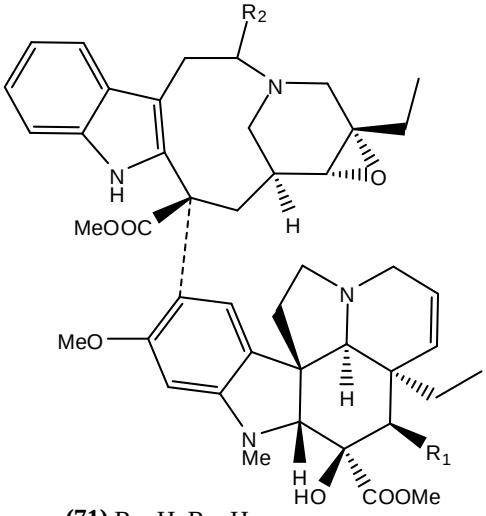
(67) C_{19}=R
(68) C_{19}=S



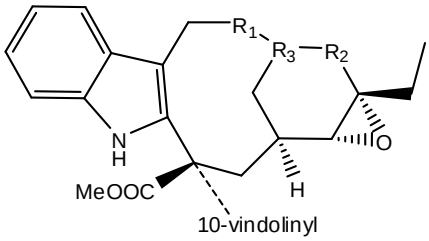
(69)



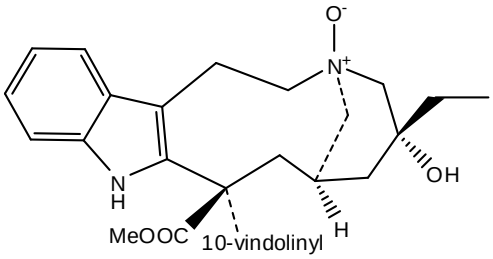
(70)



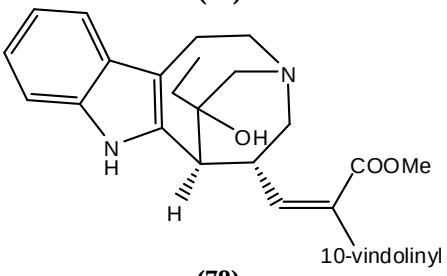
(71) R_1=H, R_2=H
(72) R_1=OAc, R_2=CH_2COCH_3



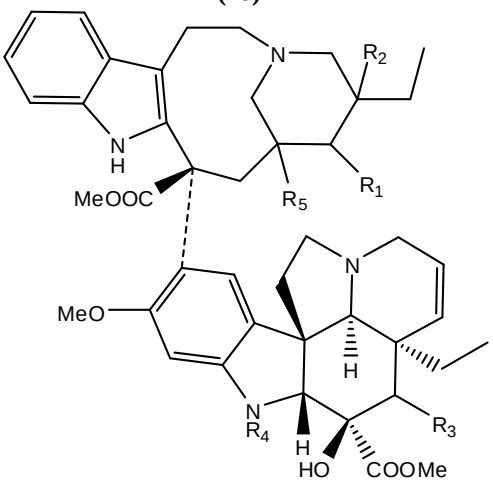
(73) R_1=CO, R_2=CH_2, R_3=N
(74) R_1=CH_2, R_2=CO, R_3=N
(75) R_1=CH_2, R_2=CH_2, R_3=N^+-O^-



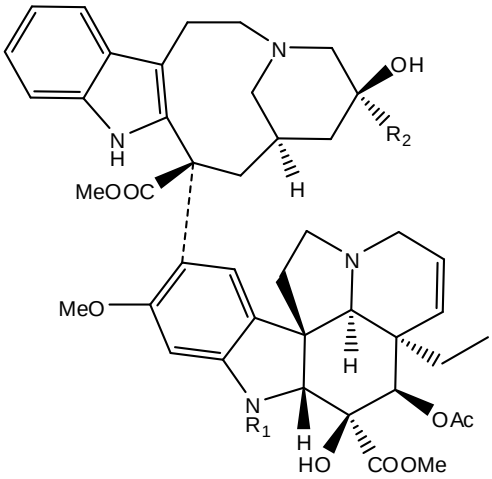
(77)



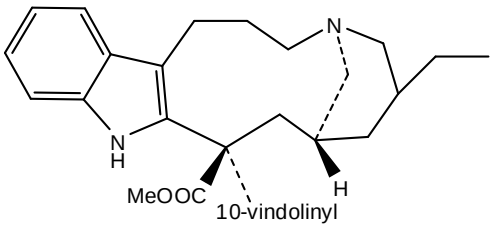
(78)



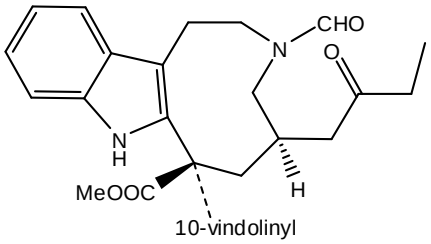
(80) R_1=OH, R_2=OH, R_3=H, R_4=Me, R_5=H
(81) R_1=H, R_2=OH, R_3=OH, R_4=Me, R_5=H
(82) R_1=H, R_2=OH, R_3=OAc, R_4=H, R_5=H
(83) R_1=H, R_2=H, R_3=OAc, R_4=Me, R_5=OH
(84) R_1=H, R_2=OH, R_3=OAc, R_4=Me, R_5=H
(85) R_1=OH, R_2=OH, R_3=OAc, R_4=Me, R_5=H
(86) R_1=OH, R_2=H, R_3=OH, R_4=Me, R_5=H



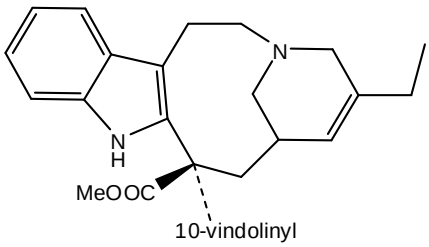
(76) R_1=Me, R_2=OH



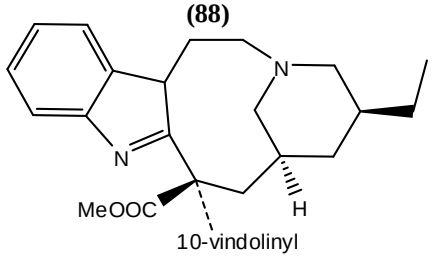
(79)



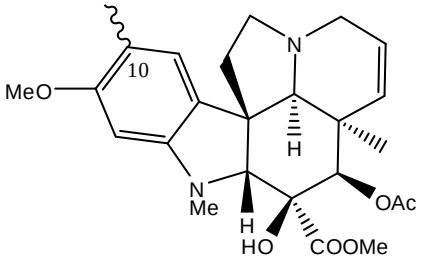
(87)



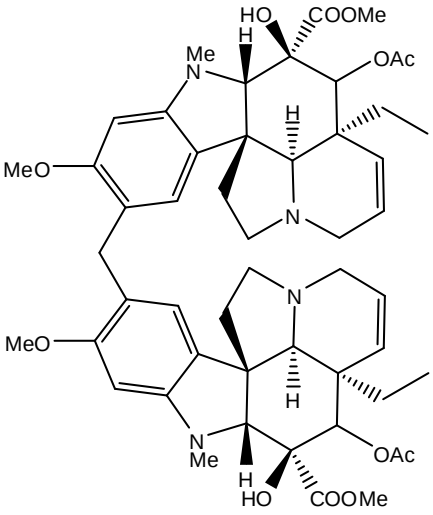
(88)



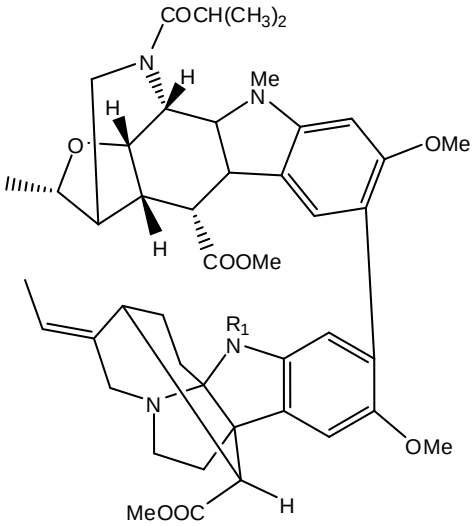
(89)



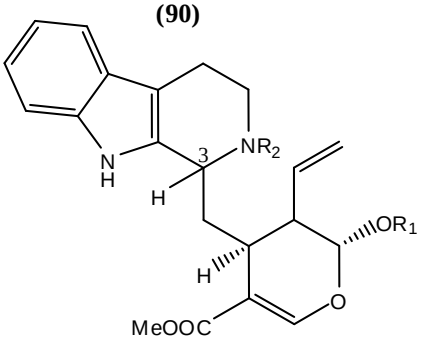
10-vindolinyl



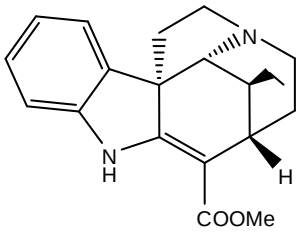
(90)



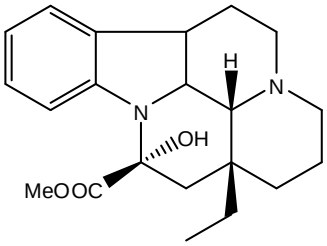
(91) R_1=H
(92) R_1=Me



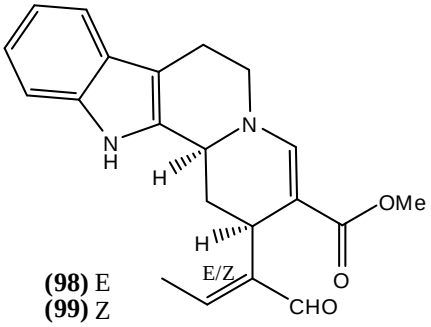
(93) C_3=*R*, R_1=Lactam, R_2=H
(94) C_3=*S*, R_1=Glucose, R_2=H
(95) C_3=*S*, R_1=Glucose, R_2=Ac



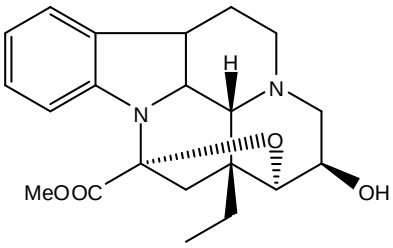
(96)



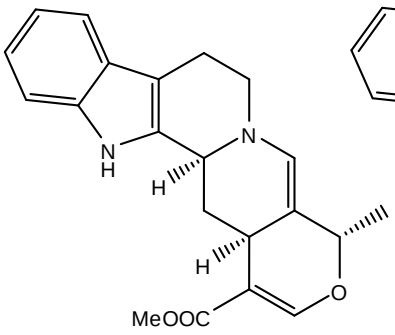
(97)



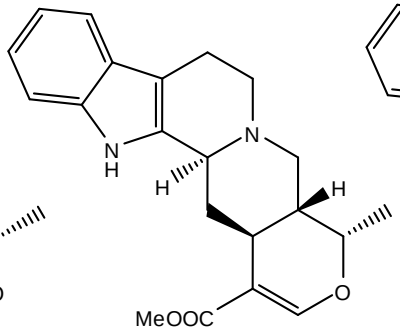
(98) E
(99) Z



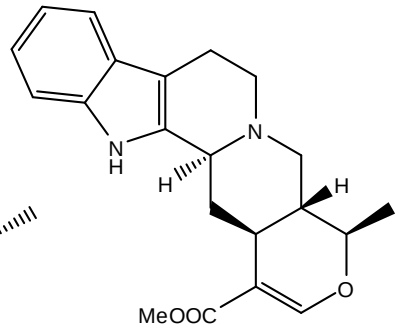
(100)



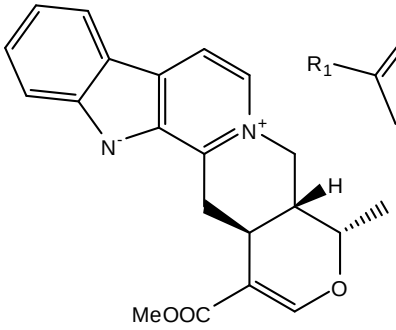
(101)



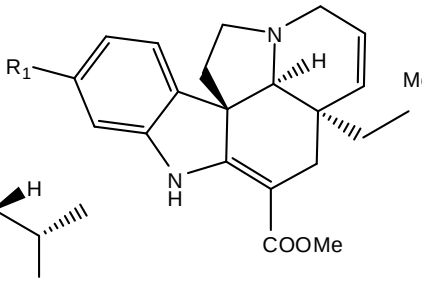
(102)



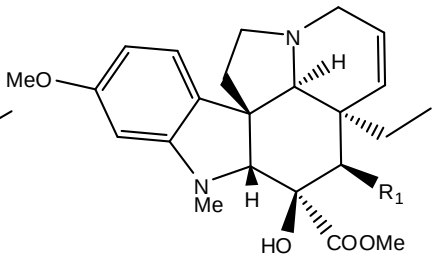
(103)



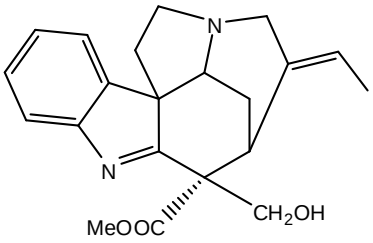
(104)



(105) R_1=H
(106) R_1=OMe



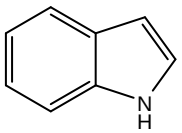
(107) R_1=H
(108) R_1=OMe



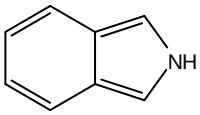
(109)

2.2 Indole Alkaloids

The indole system can be visualized as a fusion of a pyrrole ring and a benzene ring that give two isomeric benzopyrroles: Indole **(110)** and isoindole **(111)**.



(110)

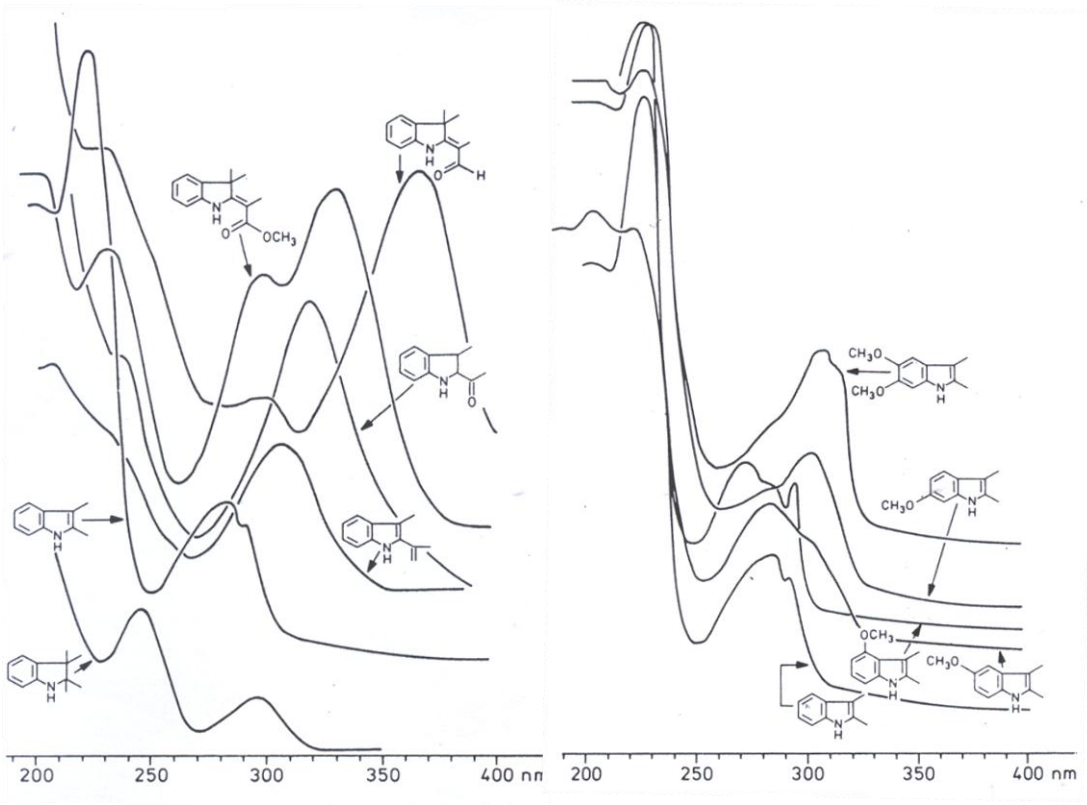


(111)

Indole **(110)** is a crystalline solid with melting point of 52°C and soluble in most organic solvents. It is a weak base with delocalized electrons from nitrogen into the

aromatic structure and can be reduced upon acid introduction to form salt. However, its true salts are unknown.[86]

Indole **(110)** consists of a chromophore that will show characteristic ultraviolet spectrum with maxima at 225 nm and 270 nm.[87] As structural change occur in chromophore, the exact energy and intensity of absorption will change. Figure 2.2 shows the UV spectra of the common indole chromophore (a) and effect of substituent to the UV spectrum of indole (b).[88]

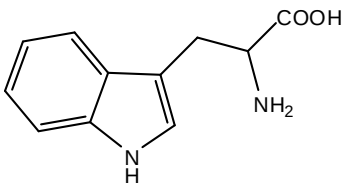


(a) (b)

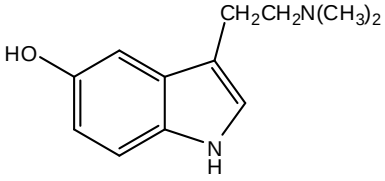
Figure 2.2: (a) UV spectra of some common indole chromophores. (b) UV spectra of some common substitution in indole chromophores.[88]

Indole alkaloids are derived from tryptophan **(112)**, their amino acid precursor. There are two main classes of indole alkaloids, both having the indole moiety from tryptophan **(112)**. The first class is the simple indole alkaloids. Simple indole alkaloids do not present a structural uniformity, having only the indole nucleus or a direct derivative of it as a common feature such as bufotenine **(113)**, gramine **(114)**, pscilocybine **(115)**, phytostigmines **(116)** and harmaline **(117)**.[89]

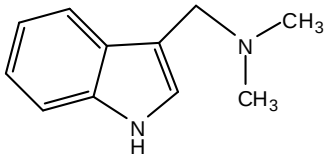
Indole bases of the second class contain two structural elements: tryptophan **(112)** or tryptamine **(118)** as the indole nucleus and a C_9- or C_{10}- monoterpene moiety derived from secologanin **(119)**. This class of indole alkaloids is known as monoterpenoid indole alkaloid.



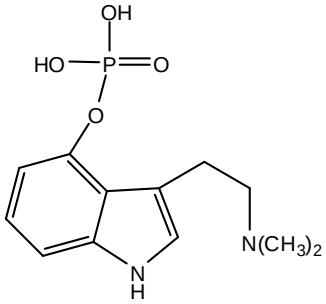
(112)



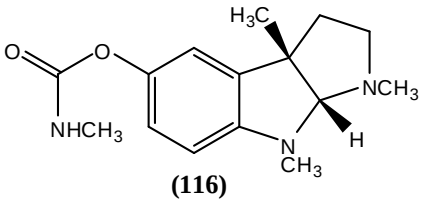
(113)



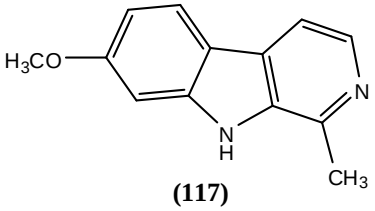
(114)



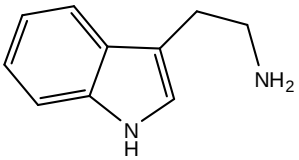
(115)



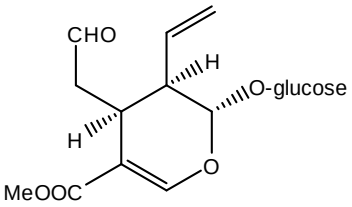
(116)



(117)



(118)



(119)

2.3 Biosynthesis of the Monoterpenoid Indole Alkaloids from *Catharanthus roseus*

Every metabolite in green plant is the product of photosynthesis, either directly or indirectly. Therefore, biosynthesis of indole alkaloids also starts from sugar production through photosynthesis. Sugar that is produced will serve as the substrate for glycolysis. In glycolysis sugar will be broken into smaller organic molecules to supply both energy by releasing chemical energy and material for biosynthesis of indole alkaloids (Figure 2.3).[90]

Part of the energy is used while some are stored with conversion of enzymatic cofactors to their more energetic state such as reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). NADPH is a strong reducing agent and carrier of chemical potential. ATP is the carrier of chemical potential and a powerful phosphorylating agent.[89]

Sugar such as glucose is broken down into phosphoenol pyruvate **(120)** and erythrose-4-phosphate **(121)** through glycolysis. Phosphoenol pyruvate **(120)** and erythrose-4-phosphate **(121)** both are used in the production of indole alkaloid precursor, tryptophan **(112)** through shikimate **(122)** and chorismate **(123)** biosynthesis (Figure 2.3 & 2.4).[91] Tryptophan **(112)** can only be synthesized by bacteria and green plants. Most indole alkaloids are derived from tryptamine **(118)**, the decarboxylation product of tryptophan **(112)** (Figure 2.3).

Phosphoenol pyruvate **(120)** is further reduced into pyruvate **(124)**. The terpenoids moiety in monoterpenoid indole alkaloids was produced by triose phosphate/pyruvate or "non-mevalonate" pathway.[92] The respective triose phosphate, glyceraldehyde-3-phosphate **(125)** was firstly produced in the Calvin cycle of photosynthesis.[93] It was used together with pyruvate **(124)** produce 1-deoxy-D-xylulose-5-phosphate **(126)** that is the precursor involve in formation of isopentenyl pyrophosphate **(127)**, the precursor of all terpenoids.[94; 95] Secolaganin **(119)** is derived from "non-mevalonate" pathway by way of isopentenyl pyrophosphate **(127)** and geraniol **(128)** (Figure 2.5).[95]

The biosynthesis of monoterpenoid indole alkaloids starts by the stereoselective condensation of tryptamine **(118)** with secologanin **(119)** to isovincoside, also known as strictosidine **(129)** (Figure 2.3).[89] Strictosidine **(129)** is futher biosynthesize to dehydrogeissoschizine **(130)** that will serve as the intermediate for formation of

corynanthean, strychnan, ibogan and plumeran type of monoterpenoid indole alkaloids. (Figure 2.3 & 2.6)

Dehydrogeissoschizine **(130)** is the central intermediate for biosynthesis of corynanthean indole alkaloids. Cathenamine **(101)** is the major product from deglycosylation of strictosidine **(129)**. Reduction of cathenamine **(101)** intermediate will give other corynanthean indole alkaloids such as tetrahydroalstonine **(5)**, ajmalicine **(102)** and 19-epi-ajmalicine **(103)**. Ajmalicine **(102)** is converted into serpentine **(104)** in plant by the present peroxidase enzyme. (Figure 2.7)

The more complex plumeran, ibogan and strychnan alkaloids are derivatives of corynanthean. Strictosidine derivative, preakuammicine **(109)** is the common precursor for plumeran, ibogan and strychnan alkaloids. Preakuammicine **(109)** is from geissochizine **(131)** but the actual mechanism and physiological precursor for preakuamicine **(109)** remains unknown.[95]

Preakuamicine **(109)** is reduced to form stemmadenine **(132)** that is rapidly consumed for rearrangement to form the acrylic ester dehydrosecodine **(133)** which serves as common intermediate for plumeran and ibogan skeletons. Tabersonine **(105)**, plumeran type alkaloid is formed from a Diels-Alder reaction(Figure 2.8).[95] In our study, we obtained monoterpenoid indole alkaloids of corynanthean and plumeran from the leaves of *Catharanthus roseus* (Figure 2.8).

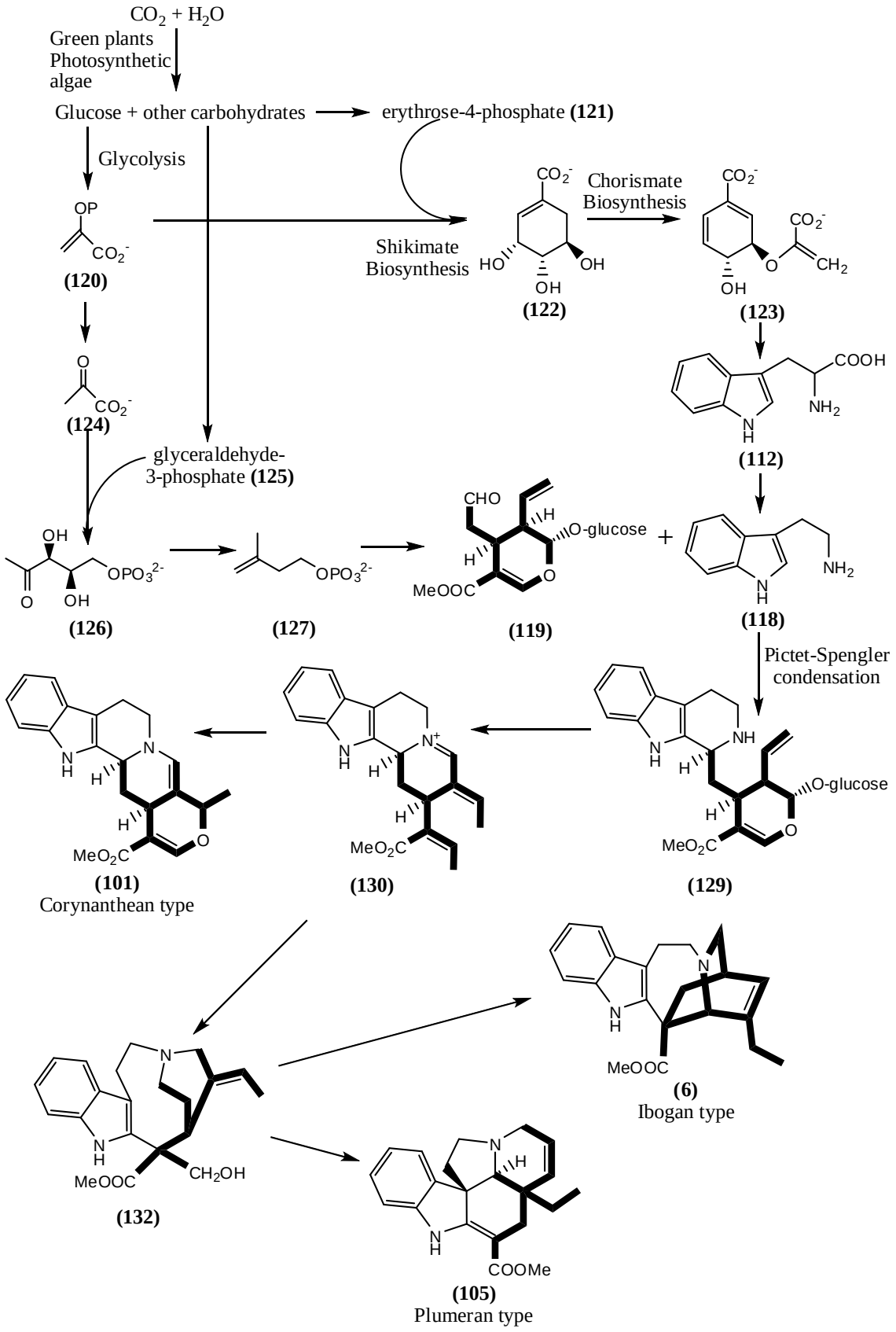


Figure 2.3: Biosynthesis of monoterpene indole alkaloids in *Catharanthus roseus*.[89-91; 96]

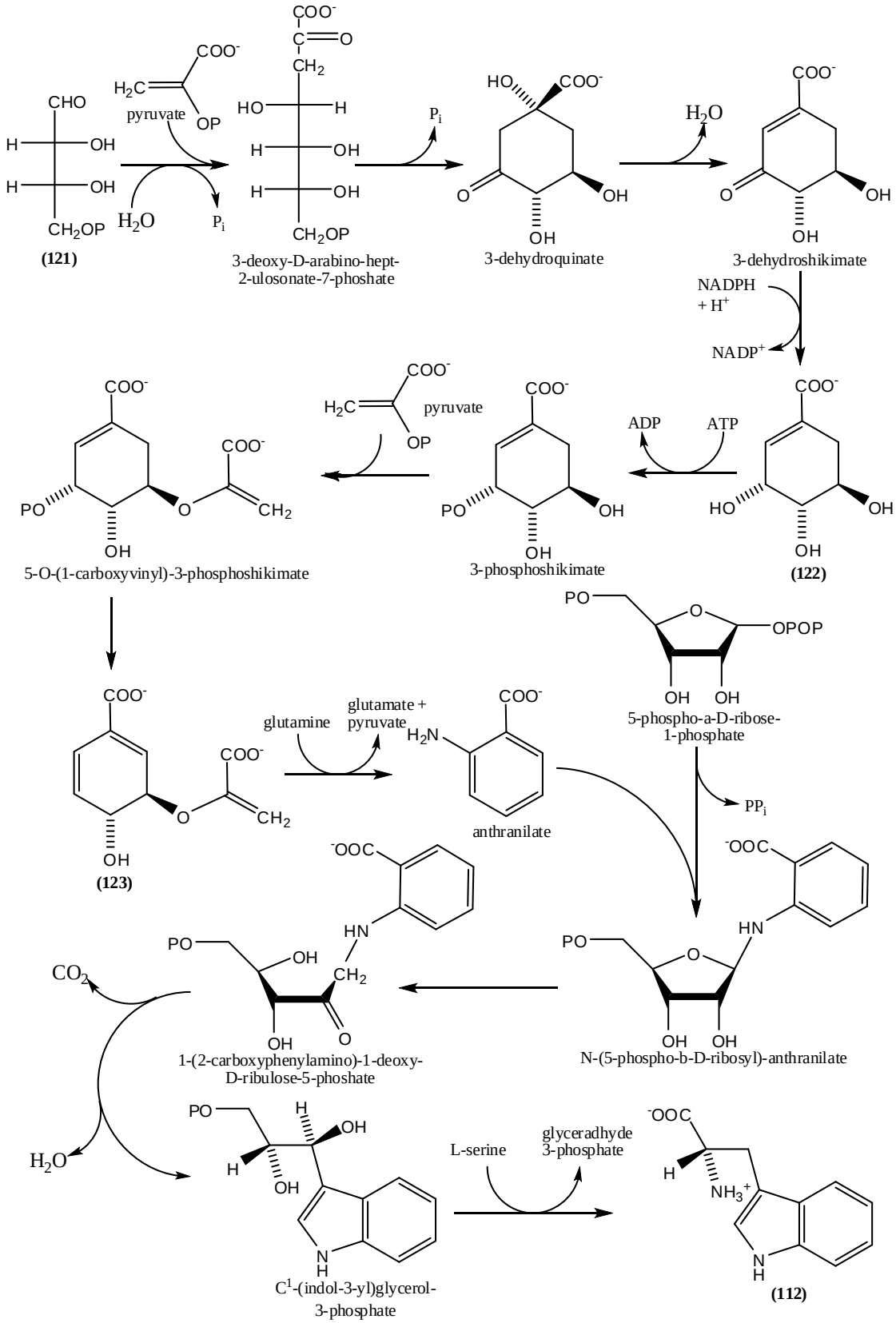


Figure 2.4: Biosynthesis of tryptophan **(112)**.[91; 96]

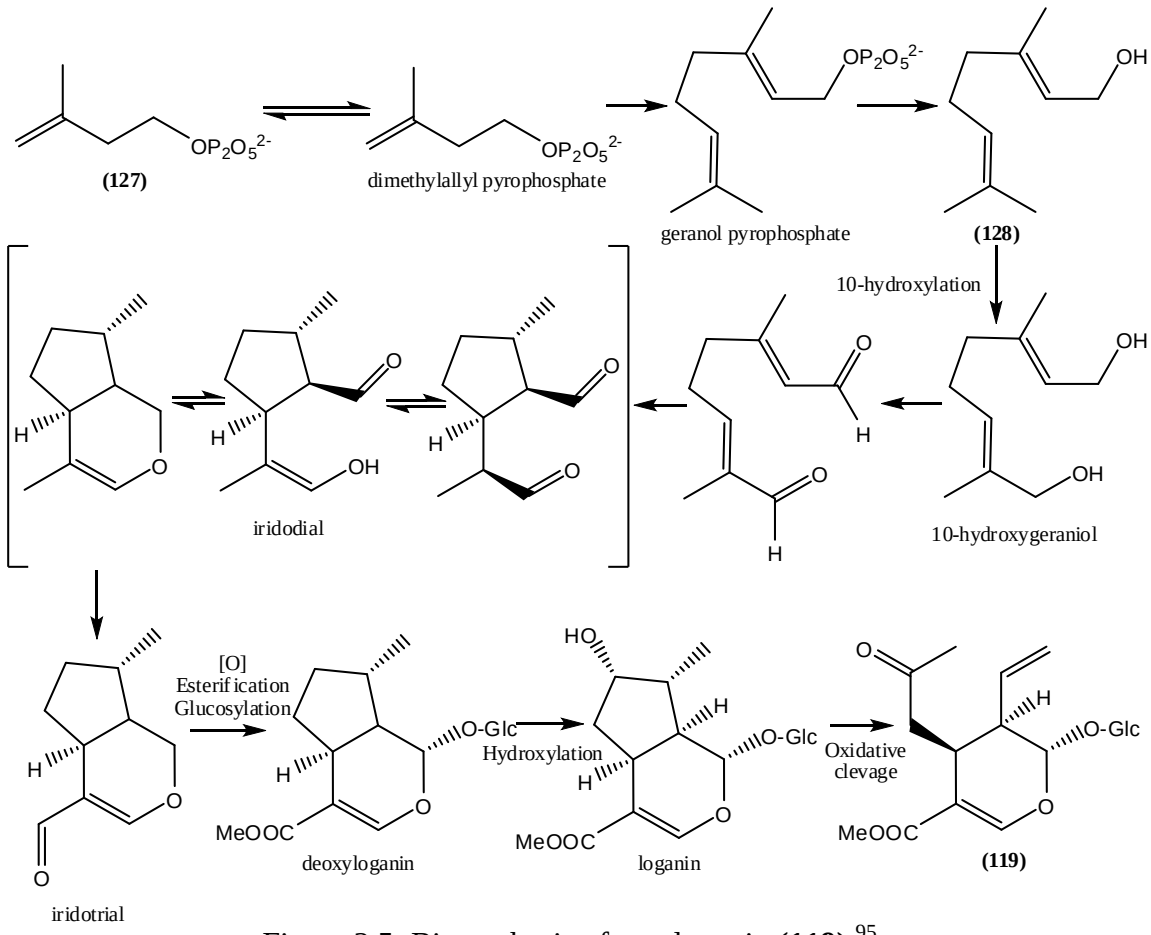


Figure 2.5: Biosynthesis of secologanin **(119)**.[95]

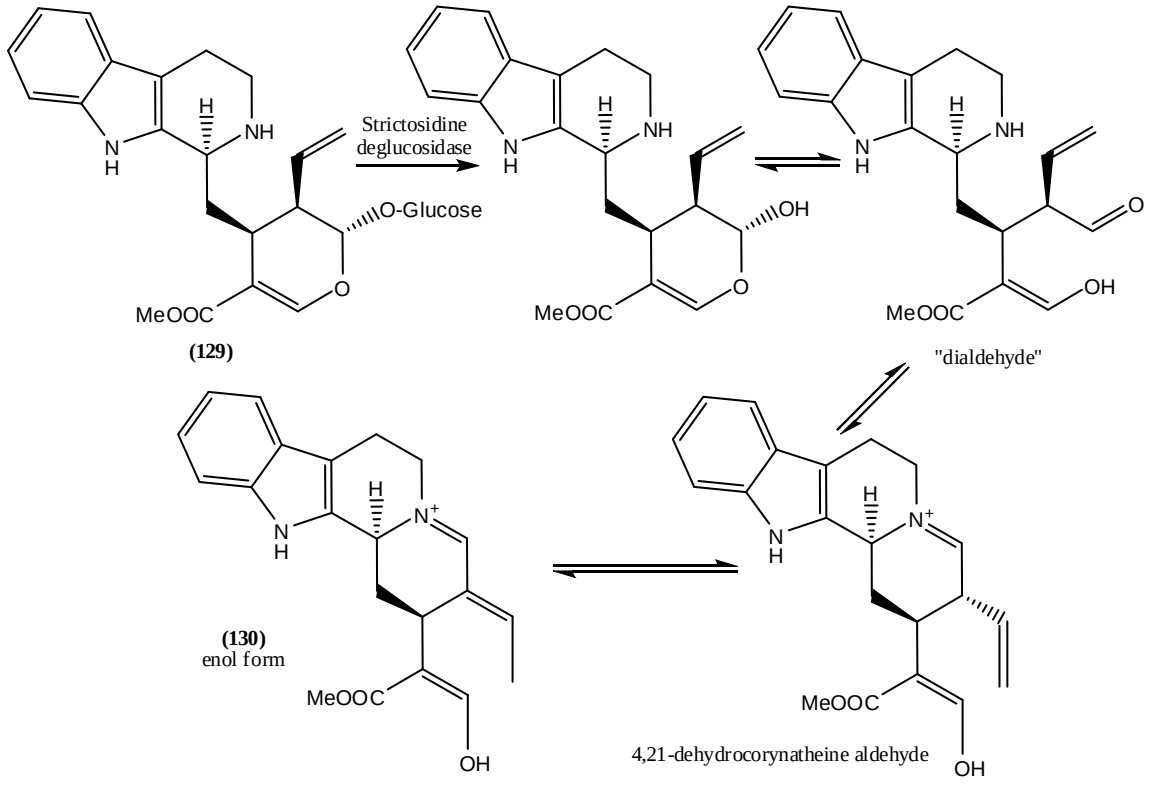


Figure 2.6: Formation of dehydrogeissoschizine **(130)** from strictosidine **(129)**.[95]

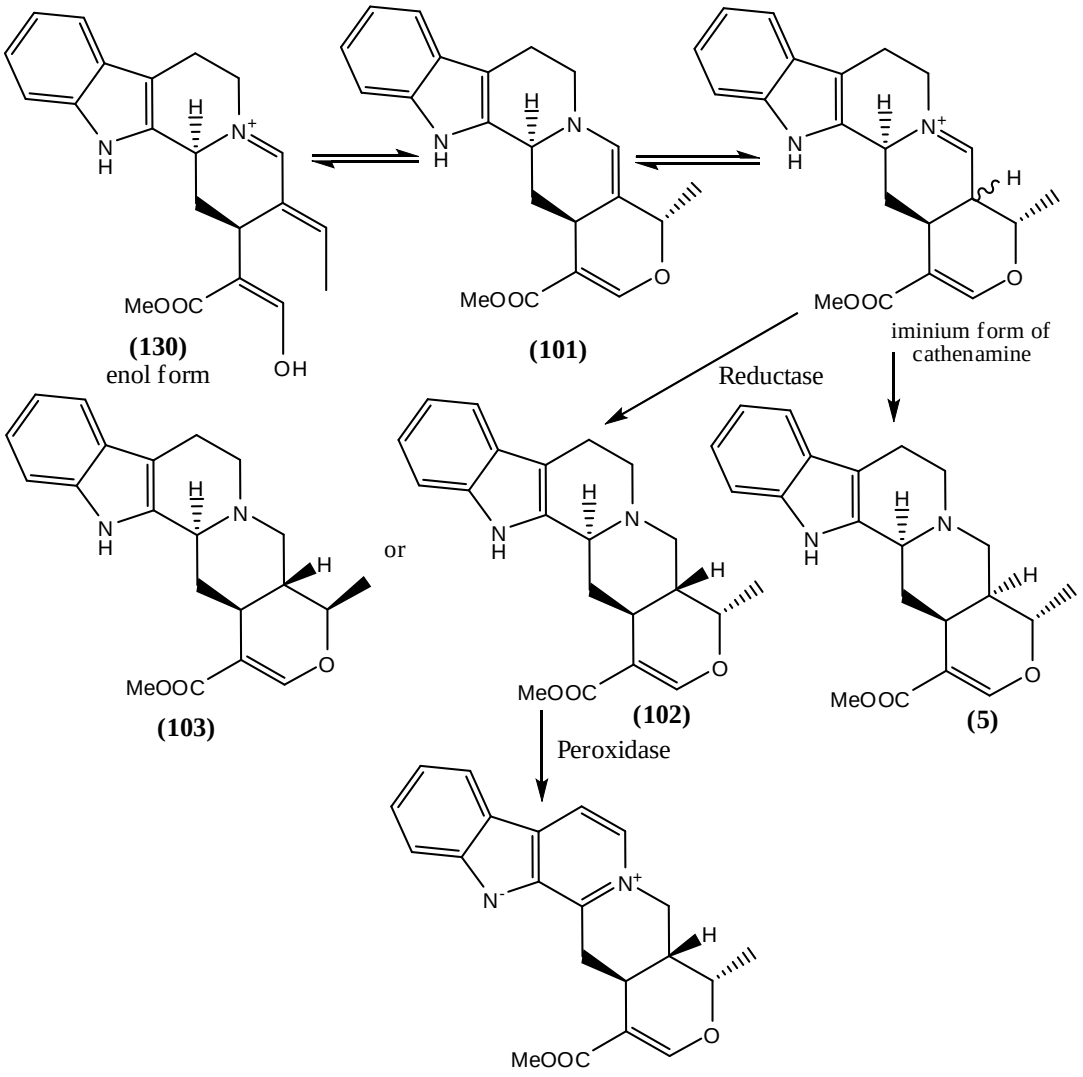
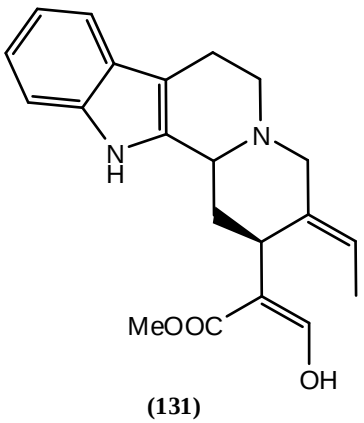


Figure 2.7: Corynanthean biosynthesis from dehydrogeissoschizine **(130)**.[95]



(131)

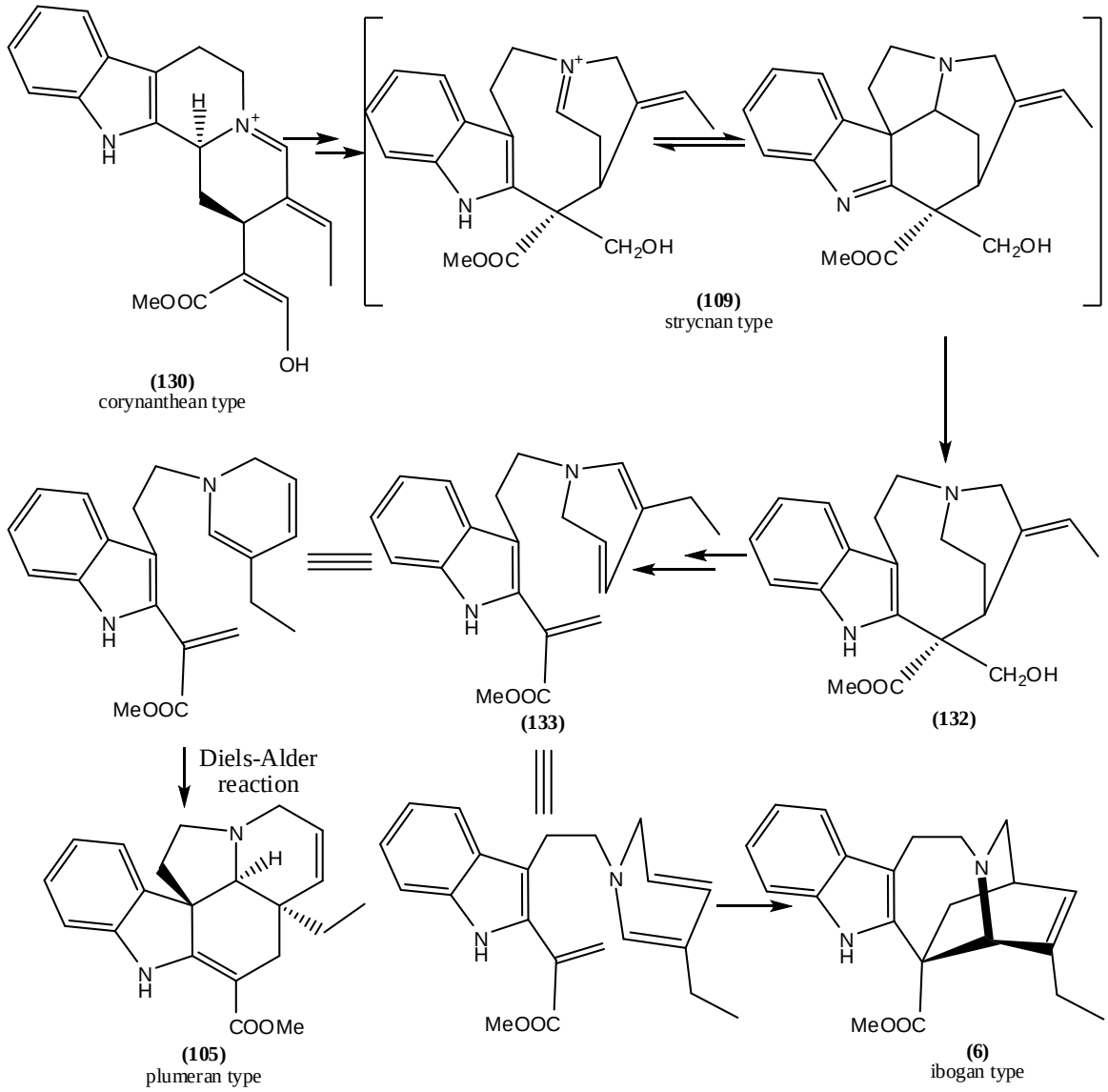


Figure 2.8: Proposed biosynthetic pathway of plumeran and ibogan alkaloids.[95]

The major indole alkaloid that was obtained in our study, vindoline **(4)** was biosynthesize from tabersonine **(105)**. Firstly, tabersonine **(105)** was hydroxylated to 11-hydroxytabersonine **(134)** followed by *O*-methylation to 11-methoxytabersonine **(106)**. Next step was hydration of a double bond to produce 11-methoxy-2,16-dihydro-16-hydroxytabersonine **(135)** followed by transfer of methyl to indole nitrogen to yield deacetoxyvindoline **(107)**. Introduction of O-acetyl moiety at C-16 was achieved by hydroxylation to yield deacetylvindoline **(108)** and acetylation to yield vindoline **(4)** (Figure 2.9).

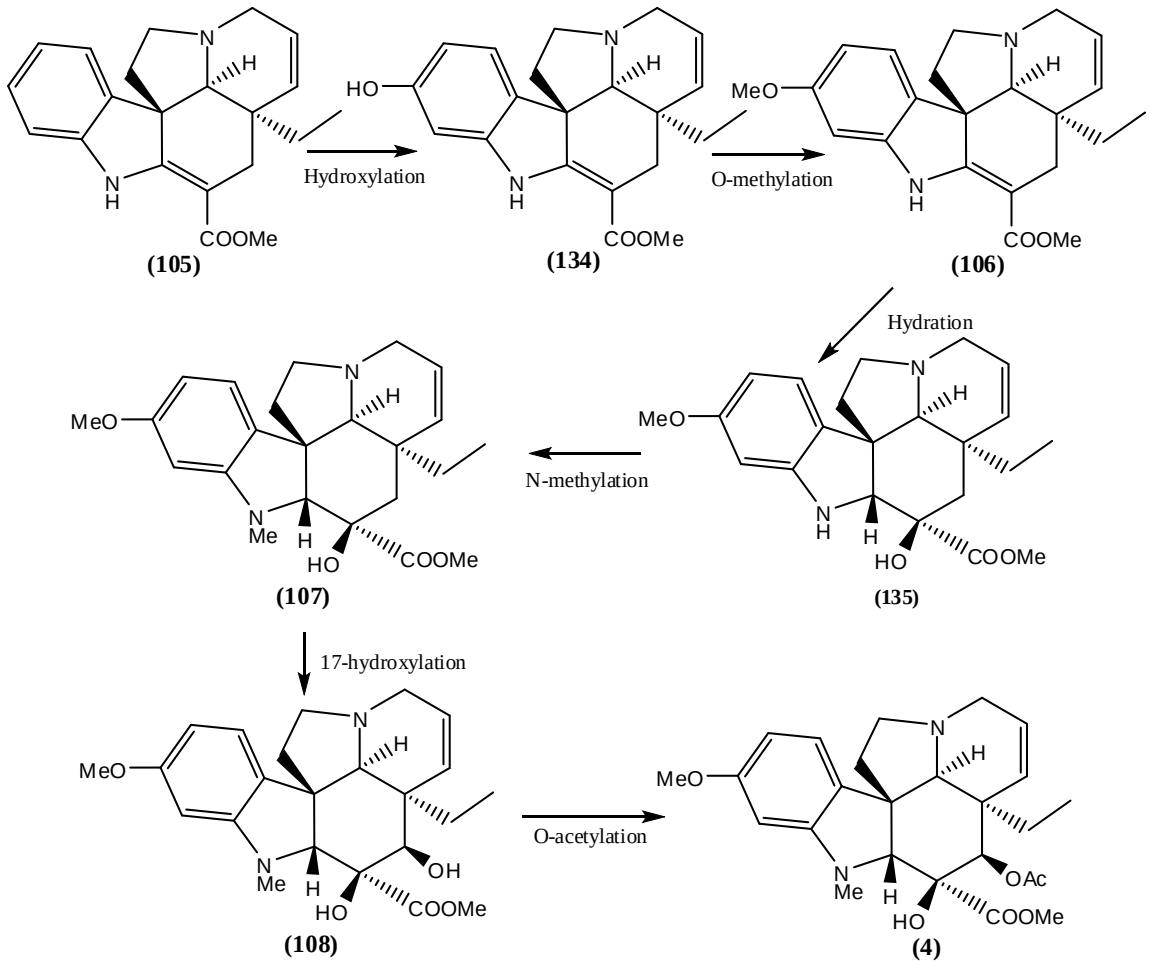


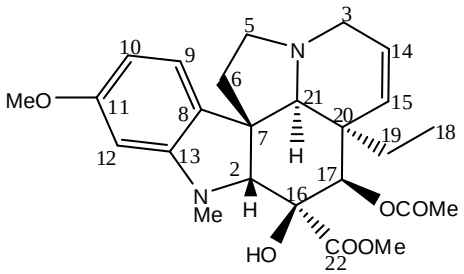
Figure 2.9: Vindoline **(4)** biosynthesis from tabersonine **(105)**.[95]

CHAPTER 3

3.1 Alkaloids isolated from *Catharanthus roseus*

The leaves of *Catharanthus roseus* (L.) G. Don. were studied for their indole alkaloids. Firstly, the leaves were de-fatted using hexane followed by general dichloromethane extraction procedure to obtain 44.5 g crude with 4.45 % yield. Next, the crude extract was subjected to acid-base extraction obtaining 6.0 g of alkaloid crude. Finally, fractionation and extensive chromatography of the alkaloid crude extract yielded seven indole alkaloids, namely: vindoline **(4)**, vindolinine **(9)**, perivine **(45)**, vindorosine **(65)**, vindolicine **(90)**, serpentine **(104)** and vindogentianine **(136)**. The following sub-chapters will discuss briefly the structural elucidation of each alkaloid.

3.2 Alkaloid I: Vindoline (4)



(4)

Alkaloid I, $[\propto]_D^{22}$ -46° (*c* 1.0, MeOH) was isolated as yellowish brown amorphous solid. Its' ESIMS spectrum showed a pseudomolecular ion peak at *m/z* 457 $(M+H)^+$ and the molecular formula $C_{25}H_{32}N_2O_6$ was established by ESI-TOFMS [*m/z* 457.2390 $(M+H)^+$, Δ +5.5 mmu]. IR absorptions implied the presence of hydroxyl (3458 cm^{-1}) and ester carbonyl (1741 cm^{-1}) functionalities.[97] Alkaloid I showed UV absorption of λ_{max}^{MeOH} $(\log \epsilon)$: 224 (5.00), 251 (4.79) and 307 (4.63) nm suggests a dihydroindole moiety[98].

The ^{13}C spectrum showed the presence of 25 carbons; three sp^3 methines, four sp^3 methylenes, five methyls, five sp^2 methines and eight carbonyl carbons. The

presence of an ester functionality was supported by the observed quaternary carbon signal at δ_C 171.9 (C-22) and the corresponding methyl resonance at δ_C 52.2 (22-OMe). The presence of an acetate functionality was indicated by the resonance at δ_C 170.8 with the corresponding methyl resonance at δ_C 21.1. Two downfield signals at δ_C 76.4 and δ_C 79.6 were due to oxygenated quaternary C-16 and oxymethine C-17 carbons respectively. Two aminomethylene carbons; C-5 and C-3 resonated at δ_C 51.9 and δ_C 51.1 while aminomethine C-21 resonated at δ_C 67.0.

The ^{1}H NMR spectrum of alkaloid I showed the presence of two strong methoxy singlets at δ_H 3.73 and δ_H 3.72 that belong to 22-OMe and 11-OMe respectively. Two strong singlets at δ_H 2.62 and δ_H 2.02 revealed the presence of N-methyl and acetyl respectively. Three singlets of two aminomethine (H-21 & H-2) and a methine (H-17) appeared at δ_H 2.61, δ_H 3.69 and δ_H 5.45 respectively. Three aromatic protons H-12, H-10 and H-9 resonated at δ_H 6.01, δ_H 6.28, δ_H 2.83 as doublet (J = 2.3 Hz), doublet of doublets (J = 2.3, 8.2 Hz) and doublet (J = 8.2 Hz) respectively. The observed splitting pattern suggested that C-11 is substituted. Two olefinic *cis* proton signals appeared at δ_H 5.18 (1H, d, J = 10.6 Hz, H-15) and δ_H 5.79 (1H, ddd, J = 1.8, 5.0, 10.6 Hz, H-14).

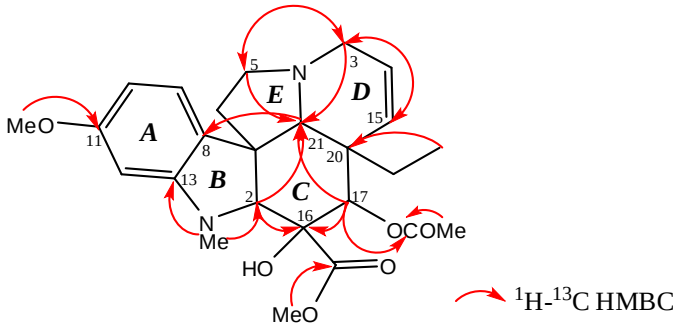
Table 3.1: ^{1}H and ^{13}C-NMR Data for Alkaloid I compared with the literature of vindoline **(4)**.[99-101]

Position	Alkaloid I		Vindoline **(4)**	
	δ_H	δ_C	δ_H	δ_C
2	3.69 (s, 1H)	83.4	3.75	83.2
3	3.44 (α, ddd, 1H$_a$, J = 1.7, 5.0, 16.5 Hz) 2.77 (β, dt, 1H$_b$, J = 1.7, 16.5 Hz)	51.1	3.40	50.9
5	3.36 (α, ddd, 1H$_a$, J = 4.6, 8.5, 14.2 Hz) 2.47 (β, dt, 1H$_b$, J = 8.5, 10.1 Hz)	51.9	2.1-3.0	51.9
6	2.25 (m, 2H)	44.1		43.9
7	-	52.8	-	52.6
8	-	125.0	-	124.9
9	6.83 (d, 1H, J = 8.2 Hz)	122.7	6.91	122.4
10	6.24 (dd, 1H, J = 2.3, 8.2 Hz)	104.6	6.30	104.5
11	-	161.1	-	161.1
12	6.01 (d, 1H, J= 2.3 Hz)	95.8	6.08	95.6
13	-	153.7	-	153.6

14	5.79 (ddd, 1H, J = 1.7, 5.0,10.6 Hz)	124.1	5.88	123.9
15	5.18 (br-d, 1H, J=10.6 Hz)	130.5	5.23	130.2
16	-	79.6	-	79.5
17	5.40 (α, s, $1H_a$)	76.4	5.43	76.2
18	0.43 (t, 3H, J = 7.3 Hz)	7.7	0.48	7.5
19	1.58 (α, q, $1H_a$, J = 7.3 Hz) 1.07 (β, q, $1H_b$, J = 7.3 Hz)	30.8	1.35	30.6
20	-	42.9	-	42.8
21	2.61 (s, 1H)	67.0	2.65	67.0
22	-	171.9	-	171.1
OCOMe	-	170.8	-	170.4
COMe	2.02 (s, 3H)	21.1	2.07	20.8
NMe	2.62 (s, 3H)	38.3	2.68	38.0
11-OMe	3.73 (s, 3H)	55.3	3.80	55.1
22-OMe	3.72 (s, 3H)	52.2	3.80	51.9
OH	9.55	-	9.00	-

The ^{1}H-^{13}C HMBC cross-peaks of H-3 to C-5 and C-21 together with H-5 to C-3 and C-21 established the connectivities of H-3, H-5 and H-21 through a sp^3 nitrogen. ^{1}H-^{13}C HMBC cross-peaks of H-15 to C-3 established the presence of olefenic moiety in ring *D* system. H-18 to C-20 correlation of ^{1}H-^{13}C HMBC suggested the connectivity of ethyl to ring *C* and *D*. ^{1}H-^{13}C HMBC correlation of H-17 to C-16 and H-2 to C-16 established the ring system C. The ^{1}H-^{13}C HMBC correlation of protons from different ring system such as H-5, H-3, H-17, H-2 to C-21 and H-21 to C-8 established the connection of ring system *A-E* as vindoline **(4)**. The correlation for protons of 11-OMe to C-11 indicated that C-11 was substituted. N-methyl presence in the indole system was confirmed through correlation of H-NMe to C-13 and H-2 to C-NMe. The connectivity of acetyl moiety to C-17 in ring *C* was confirmed through the correlation observed between H-17 and C-OCOMe.

Finally thorough analysis of 1D and 2D NMR (COSY, HMQC and ^{1}H-^{13}C HMBC) with coupled with comparison literature values.[99-101] It was confirmed the identity of alkaloid I as the known plumeran indole alkaloid, vindoline **(4)**.



(4)

Figure 3.1: Selected ^{1}H-^{13}C HMBC correlation of alkaloid I.

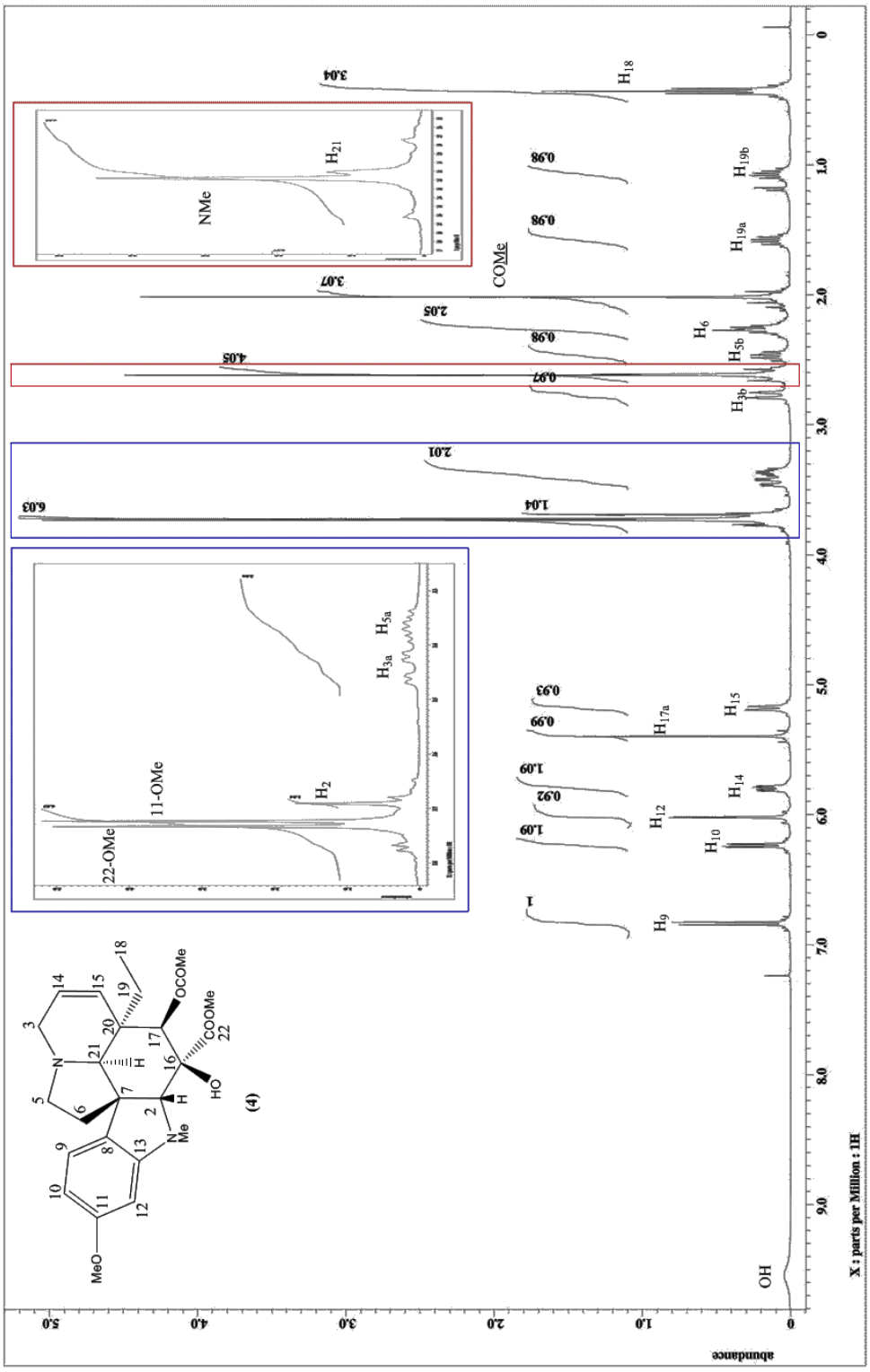


Figure 3.2: ^{1}H-NMR spectrum of alkaloid I

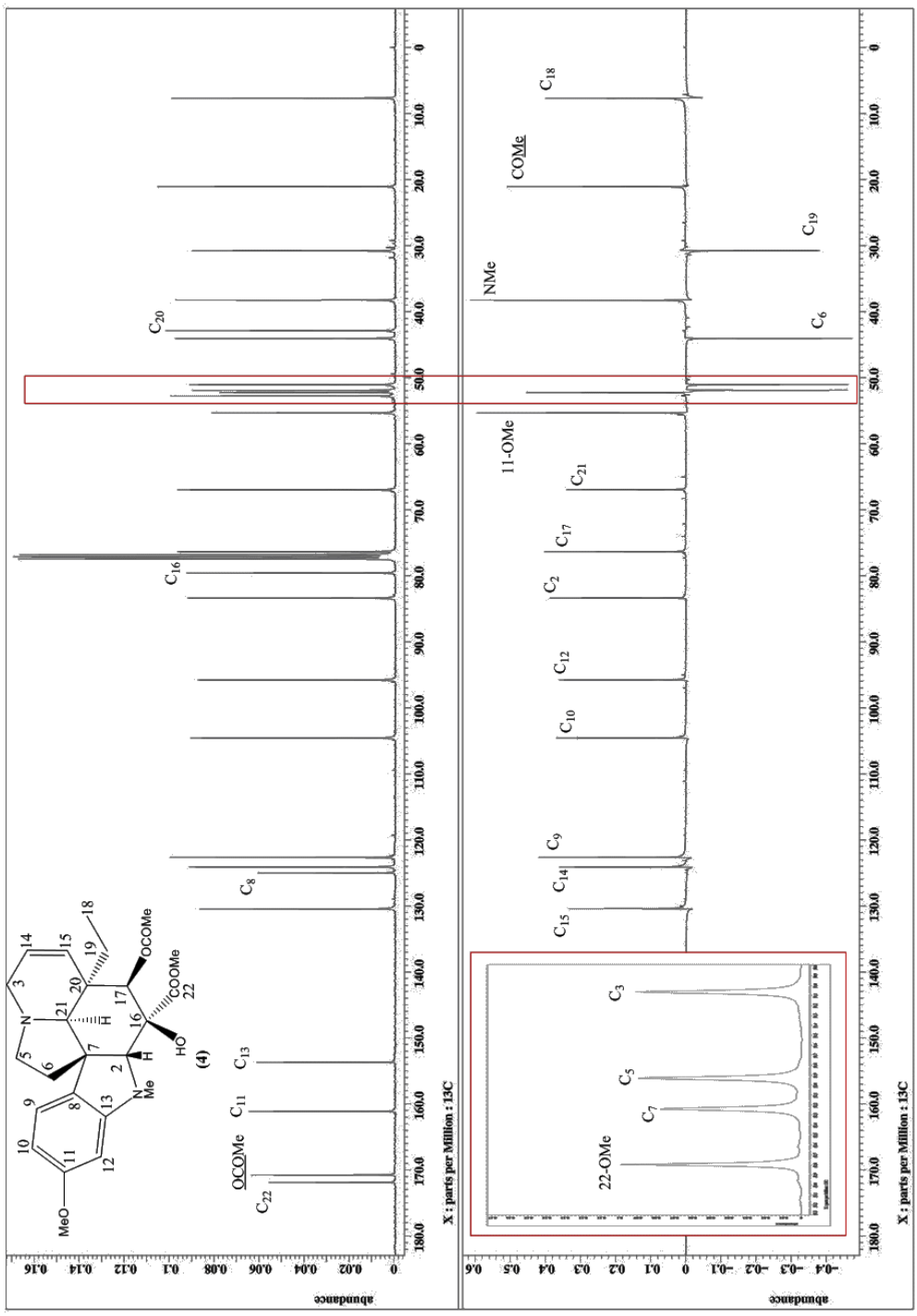


Figure 3.3: ^{13}C and DEPT-NMR spectrum of alkaloid I

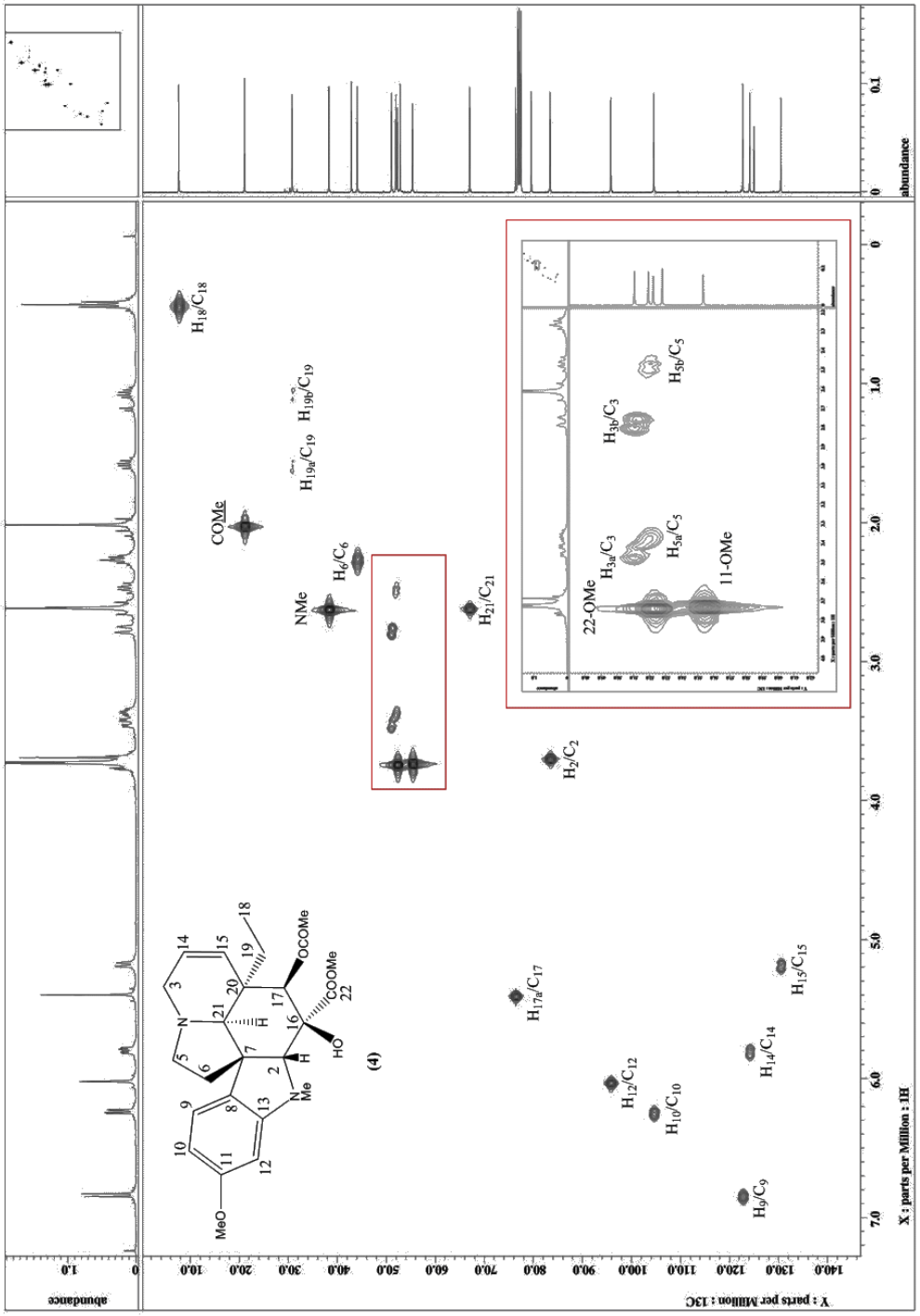


Figure 3.4: HMQC-NMR spectrum of alkaloid I

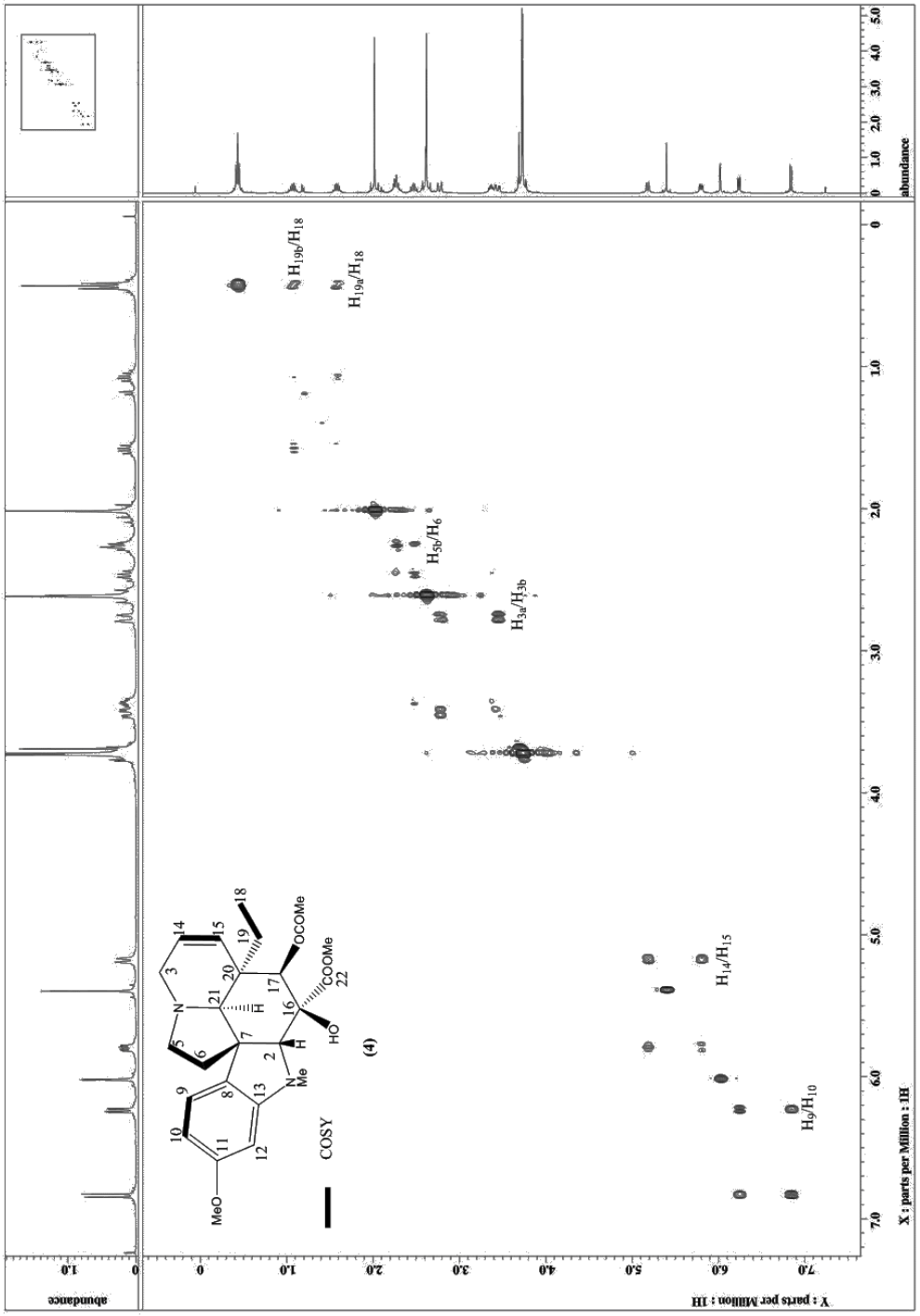


Figure 3.5: COSY-NMR spectrum of alkaloid I

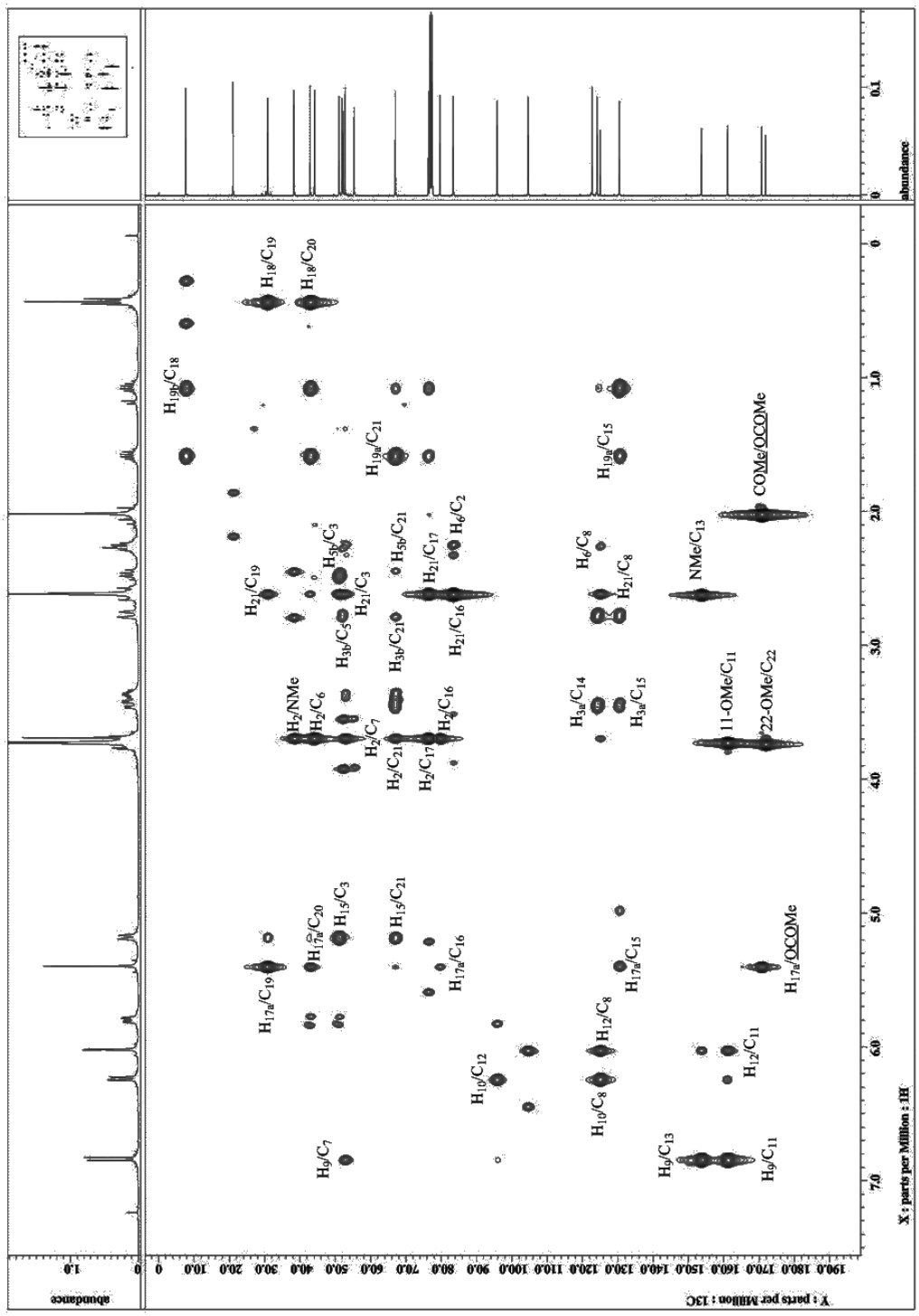
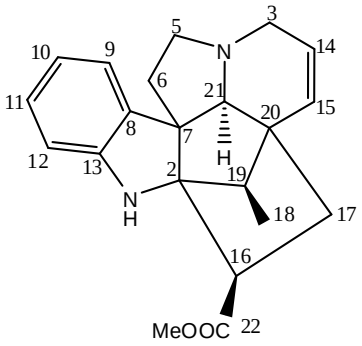


Figure 3.6: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid I

3.3 Alkaloid II: Vindolinine (9)



(9)

Alkaloid II, $[\propto]_D^{22}$ -46° (*c* 1.0, MeOH) was obtained as yellowish brown amorphous solid. The ESIMS spectrum showed a pseudomolecular ion peak at *m/z* 337 $(M+H)^+$ and the molecular formula $C_{21}H_{24}N_2O_4$ was established by ESI-TOFMS [*m/z* 337.1940 $(M+H)^+$, Δ +2.8 mmu]. IR absorptions implied the presence of amine (3365 and 1246 cm^{-1}), ester carbonyl (1725 cm^{-1}) and aromatic (1466 cm^{-1}) functionalities.[97] Alkaloid II showed UV absorption of λ_{max}^{MeOH} (log ϵ) : 216 (4.14), 244 (3.95), 295 (3.77) and 308 (3.71) nm revealed the presence of typical dihydroindole moiety.[98]

Analysis of the ^1H and ^{13}C NMR data (Table 3.2) and the HMQC spectrum of alkaloid II revealed the presence of three sp^3 methine, four sp^3 methylene, two methyl, six sp^2 methine and six quaternary carbons. The presence of an ester functionality was supported by the observed quaternary signal at δ_C 174.6 (C-22) and the corresponding methyl resonance at δ_C 52.3 (22-OMe). Two aminomethylene carbons of C-5 and C-3 resonated at δ_C 58.1 and δ_C 50.1 respectively. The aminomethine (C-21) resonated at δ_C 77.4.

The ^1H-NMR spectrum of alkaloid II showed one strong methoxyl singlet at δ_H 3.69 that belongs to 22-OMe. One singlet most probably belongs to the aminomethine H-21, was observed at δ_H 3.49. Four aromatic protons H-9, H-10, H-11 and H-12 resonated at δ_H 7.30, δ_H 6.84, δ_H 7.05 and δ_H 6.76 as doublet (J=6.7 Hz), ddd (J=1.2,

6.7, 7.4 Hz), ddd (J=1.2, 7.4, 8.0 Hz) and a doublet (J=8.0 Hz) respectively, thus, suggesting an unsubstituted indole system. A doublet (J=6.7 Hz) representing a methyl protons was observed at δ_H 0.94 (H-18). Two olefinic *cis* proton signals appeared at δ_H 6.15 (1H, dd, J = 3.6, 9.8 Hz, H-15) and δ_H 5.75 (1H, ddd, J = 1.8, 4.9, 9.8 Hz, H-14).

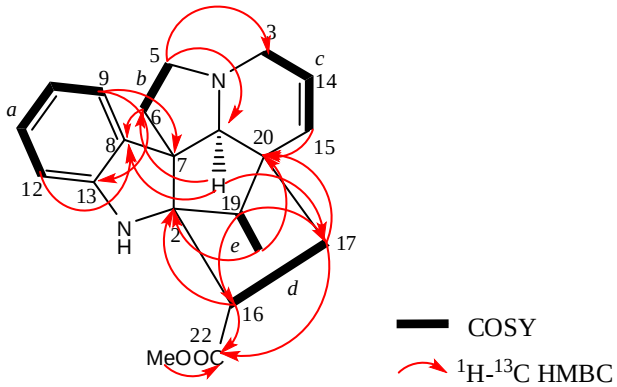
Table 3.2: ^{1}H and ^{13}C-NMR Data for Alkaloid II compared with literature of vindolinine **(9)**.[102]

Position	Alkaloid II		Vindolinine **(9)**[102]
	δ_H	δ_C	δ_H
2	-	81.6	-
3	4.00 (dd, 1H, J=4.6, 17.7 Hz) 3.46 (m, 1H overlapped with H_5 & H_{21})	50.1	3.97 (dd, 18 & 5 Hz) 3.45 (ddd, 2, 3.3& 18 Hz)
5	3.40 (m, 1H overlapped with H_3 & H_{21}) 3.30 (m, 1H)	58.1	3.40 (dd, 8 & 9 Hz) 3.29 (ddd, 7, 9 & 10.5 Hz)
6	2.15 (m, 1H) 1.87 (m, 1H overlapped with H_{17})	36.2	2.17 (dd, 7 & 16 Hz) 1.85 (ddd, 9, 10.5 & 16 Hz)
7	-	60.1	-
8	-	139.2	-
9	7.30 (d, 1H, J=6.7 Hz)	124.3	6.7-7.4
10	6.84 (ddd, 1H, J=1.2, 6.7, 7.4 Hz)	121.6	
11	7.05 (ddd, 1H, J=1.2, 7.4, 8.0 Hz)	127.7	
12	6.76 (d, 1H, J=8.0 Hz)	112.9	
13	-	149.5	
14	5.75 (ddd, 1H, J=1.8, 4.6, 9.8 Hz)	128.0	5,78 (ddd, 2, 5 & 10 Hz)
15	6.15 (dd, 1H, J=3.6, 9.8 Hz)	131.2	6.16 (dd, 3.3 & 10 Hz)
16	3.04 (dd, 1H, J=6.1, 12.2 Hz)	39.4	3.04 (dd, 6 & 12 Hz)
17	2.45 (ddd, 1H, J=1.8, 6.1, 14.6 Hz) 1.80 (m, 1H, overlapped with H_6)	29.2	2.47 (dd, 6 & 14.5 Hz) 1.80 (dd, 12 & 14.5 Hz)
18	0.94 (d, 3H, J=6.7 Hz)	7.6	0.96 (d, 7 Hz)
19	2.07 (dd, 1H, J=1.2, 6.7 Hz)	48.8	2.08 (q, 7 Hz)
20	-	46.1	-
21	3.49 (s, 1H)	77.4	3.46 (s)
22	-	174.6	
22-OMe	3.69 (s, 3H)	52.3	3.70 (s)

The structure of alkaloid II was deduced from analyses of the 2D NMR data, including COSY, HMQC and ^{1}H-^{13}C HMBC spectra in chloroform-*d* (Figure 3.21). The COSY and HMQC spectra revealed connectivities of five partial structures *a*-*e*; *a* (H-9

to H-12), *b* (H-5 to H-6), *c* (H-3 to H-15), *d* (H-16 to H-17) and *e* (H-19 to H-20) (Figure 3.7).

The ^{1}H-^{13}C HMBC cross-peaks of H-12 to C-8, H-6 to C-8 revealed the connectivity of partial structure *a* with *b* and the attachment of partial structure *b* to the indole moiety, while the ^{1}H-^{13}C HMBC cross-peaks of H-21 to C-6, C-8 and C-17 revealed the connectivity of partial structure *a, b, d*. The ^{1}H-^{13}C HMBC cross-peak of H-5 to C-3 is a direct connection of partial structure b with c, while cross-peaks of H-15, H-17, H-18 to C-20 revealed the connection of *c, d, e*. The ^{1}H-^{13}C HMBC cross-peaks of H-16 to C-2, C-22, H-18 to C-22 and 22-OMe protons to C-22 revealed the attchement of partial structure *d* and *e* with indole moiety and connectivity of the methoxy carboxylate with partial structure *d*. Thus the structure of alkaloid II was assigned as shown in Figure 3.7.



(9)

Figure 3.7: COSY and selected ^{1}H-^{13}C HMBC correlation of alkaloid II

Therefore, the COSY and ^{1}H-^{13}C HMBC correlation as shown in Figure 3.7 together with comparison of ^{1}H data of the literature value[102] confirmed the identity of alkaloid II as the known plumeran indole alkaloid, vindolinine **(9)**.

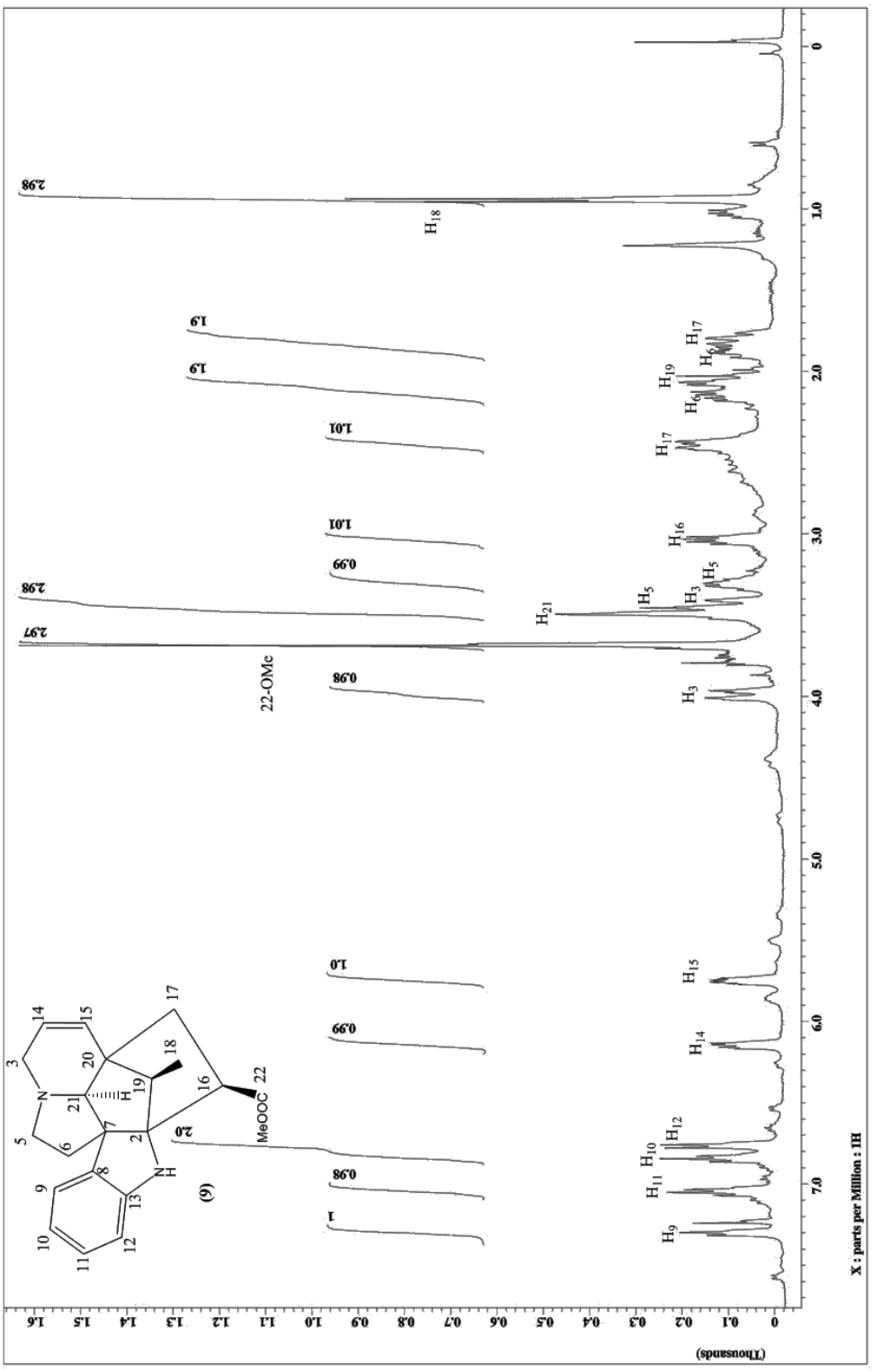


Figure 3.8: ^{1}H-NMR spectrum of alkaloid II

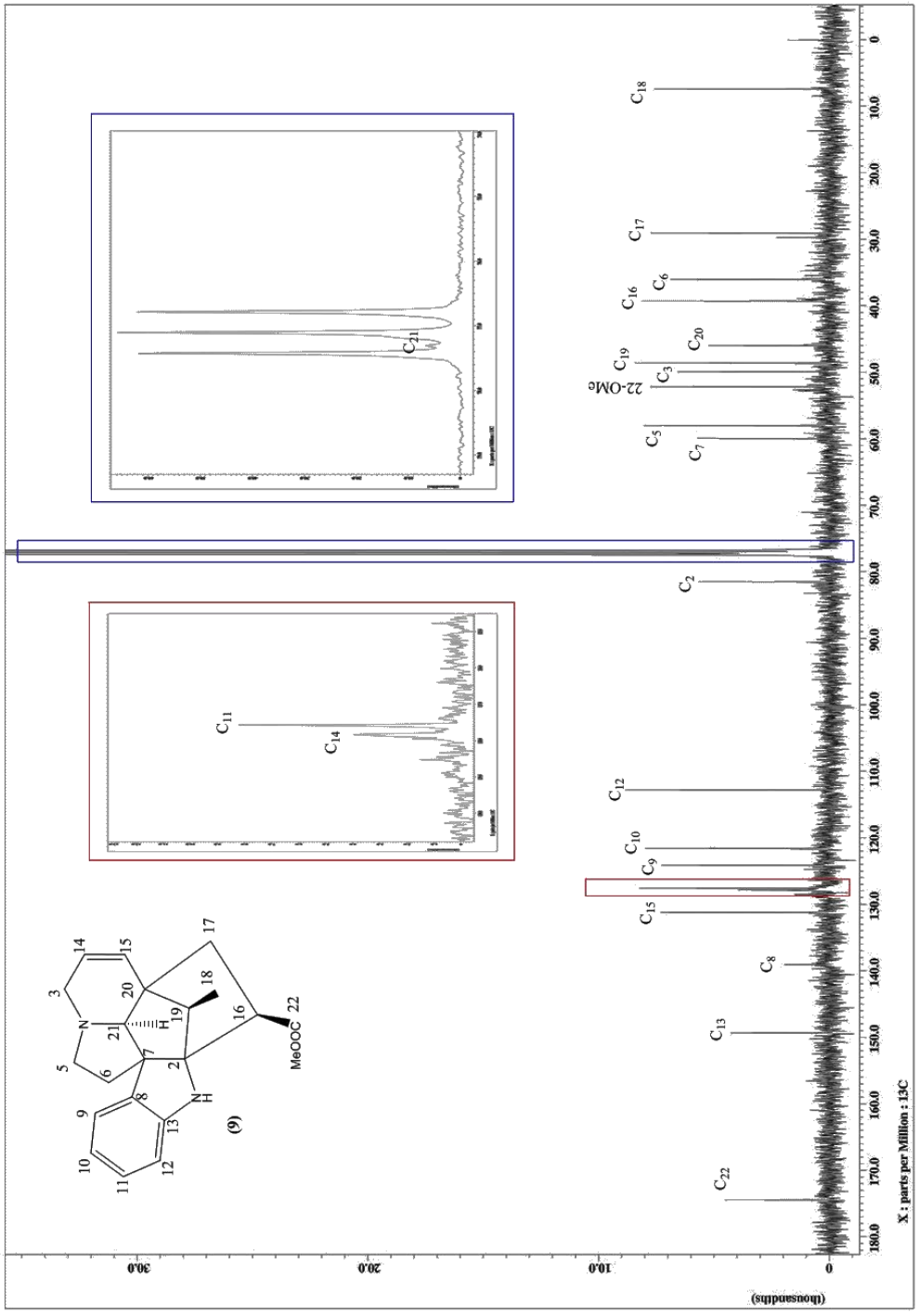


Figure 3.9: ^{13}C-NMR spectrum of alkaloid II

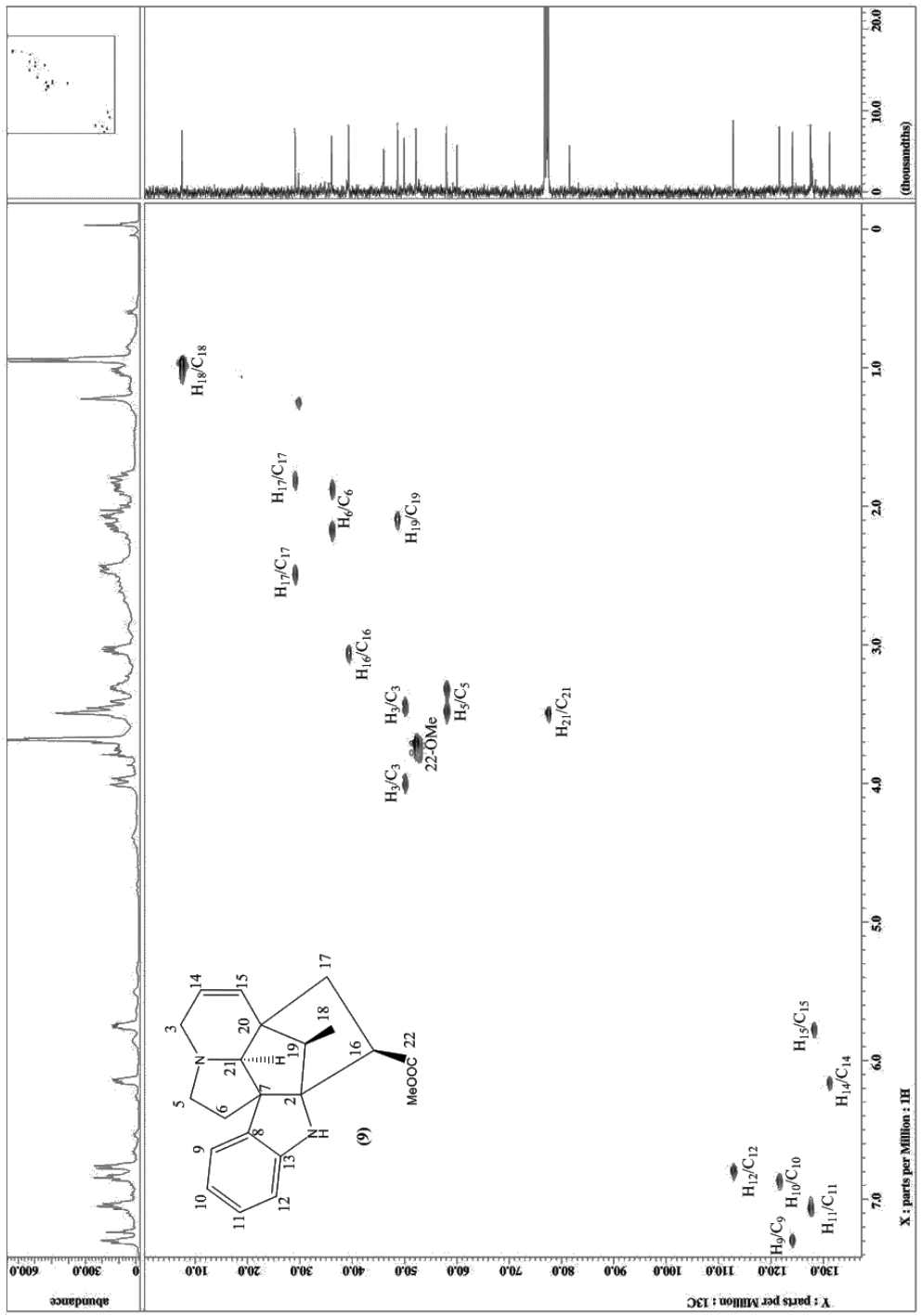


Figure 3.10: HMQC-NMR spectrum of alkaloid II

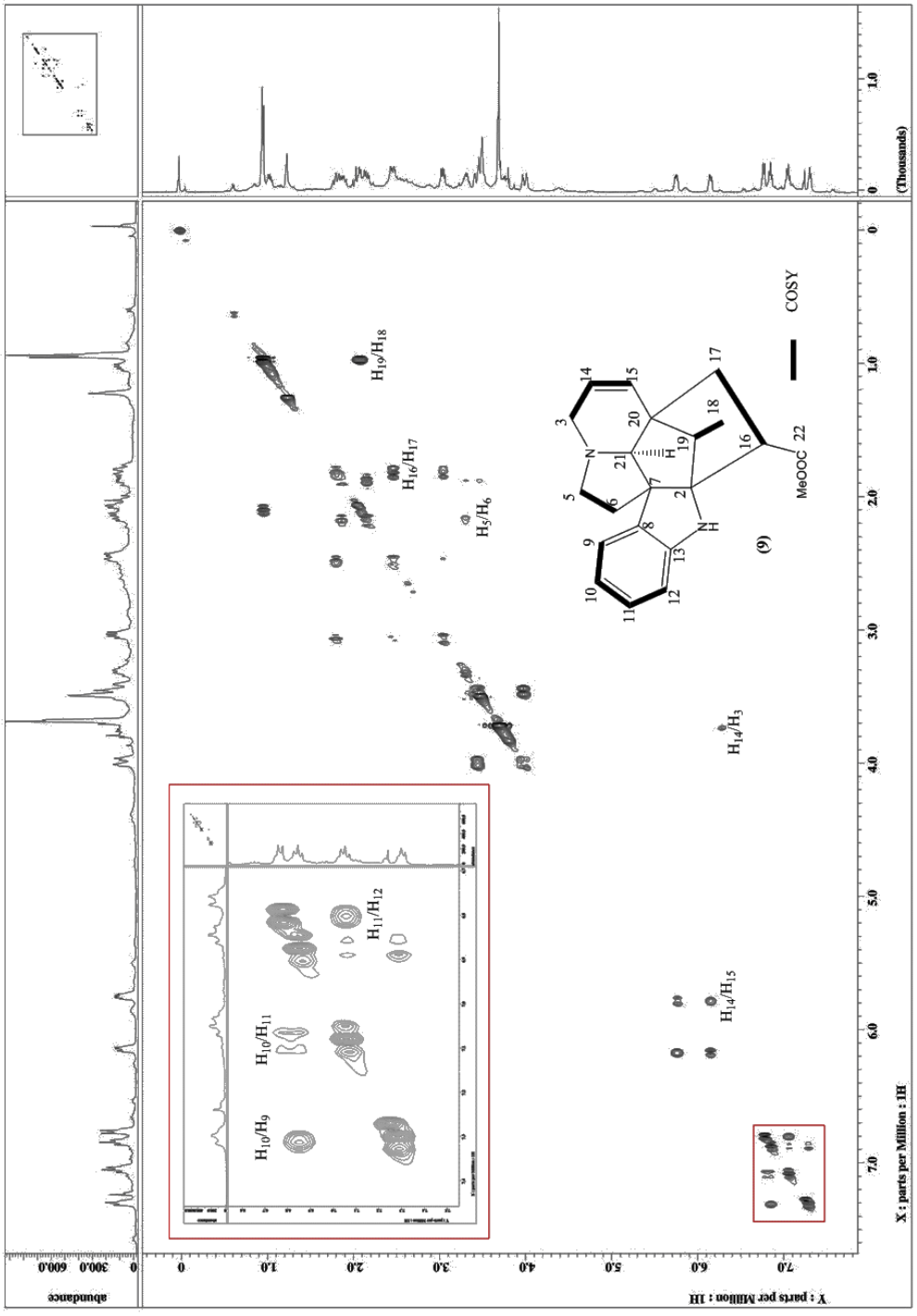


Figure 3.11: COSY-NMR spectrum of alkaloid II

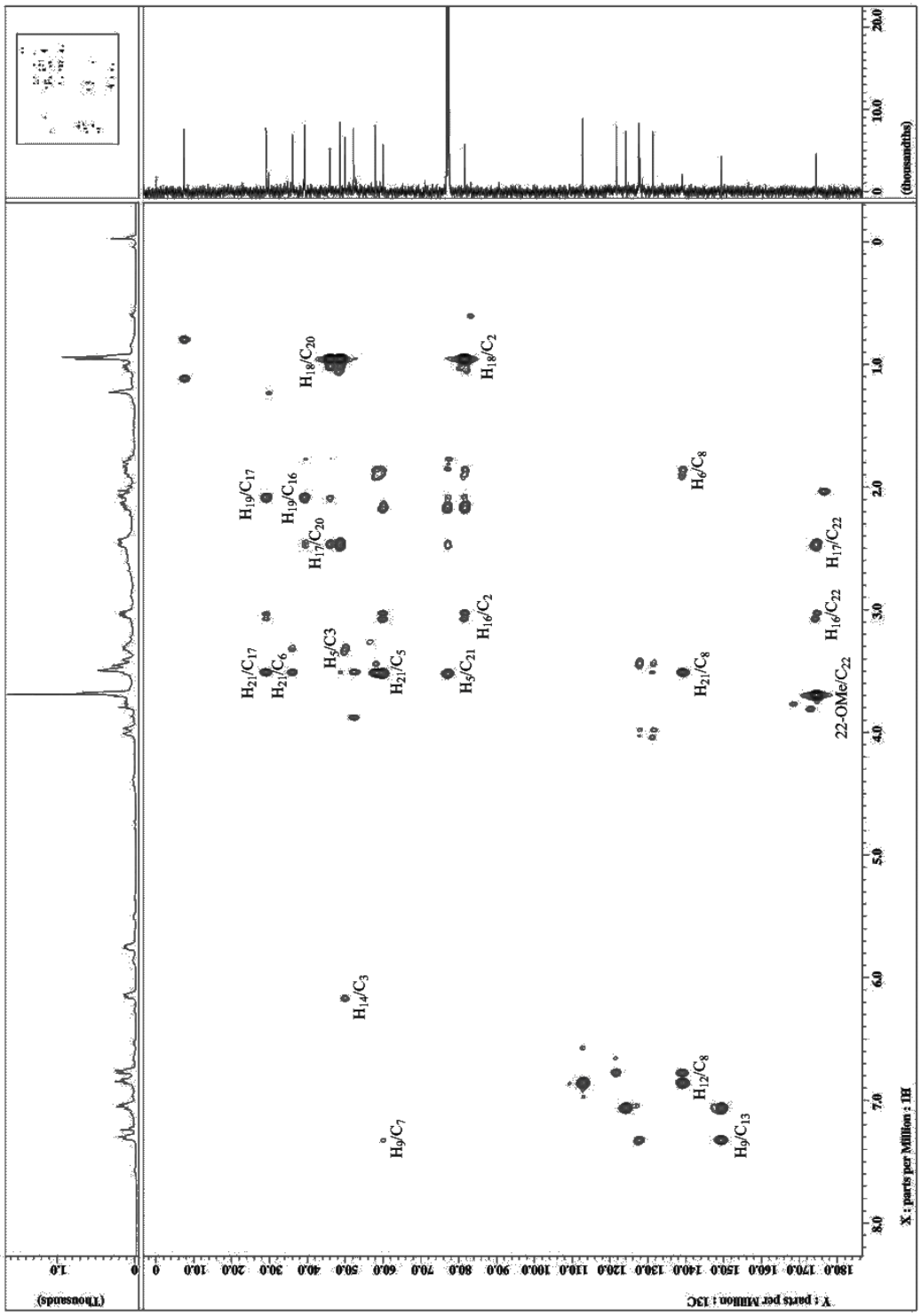
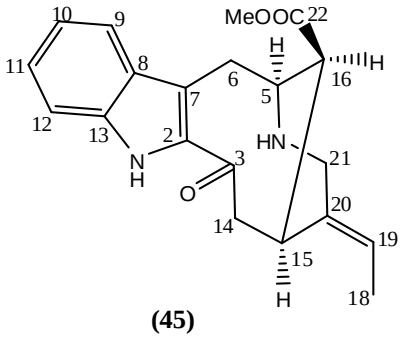


Figure 3.12: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid II

3.4 Alkaloid III: Perivine (45)



(45)

Alkaloid III, $[\propto]_D^{22}$ -76° (*c* 1.0, MeOH) obtained as yellowish brown amorphous solid. The ESIMS showed a pseudomolecular ion peak at *m/z* 339 $(M+H)^+$ and the molecular formula $C_{20}H_{22}N_2O_3$ was established by ESI-TOFMS [*m/z* 339.1690 $(M+H)^+$, Δ -1.7 mmu]. The IR absorptions implied the presence of amine (3311 and 1643 cm^{-1}) and ester carbonyl (1725 cm^{-1}) functionalities.[97] Alkaloid III showed UV absorption of λ_{max}^{MeOH} (log ϵ) : 228 (4.36), 296 (4.11) and 314 (4.29) nm.

Analysis of the ^1H and ^{13}C NMR data (Table 3.3) and the HMQC spectrum of alkaloid III revealed the presence of three sp^3 methine, three sp^3 methylene, two methyl, five sp^2 methine and seven quaternary carbons. The presence of an ester functionality was supported by the observed quaternary signal at δ_C 171.2 (C-22) and the corresponding methoxy resonance at δ_C 50.7 (22-OMe). A quaternary ketone carbon signal that resonated at δ_C 190.5 (C-3) was also observed. Two downfield signals of an aminomethine C-5 and aminomethylene C-21 carbons were found resonated at δ_C 51.0 and δ_C 25.7 respectively. Two signals for olefinic quaternary C-20 and methine C-19 carbons were observed at δ_C 137.8 and δ_C 120.4 respectively.

^1H-NMR spectrum of alkaloid III revealed the presence of one methoxy signal for 22-OMe at δ_H 2.62, a doublet signal of methyl for H-18 at δ_H 1.67 and an olefinic proton H-19 signal at δ_H 5.41. It also revealed an unsubstituted aromatic indole system

with the presence of four aromatic proton signals at δ_H 7.13 (H-10), δ_H 7.32 (H-11 & H-12) and δ_H 7.70 (H-9).

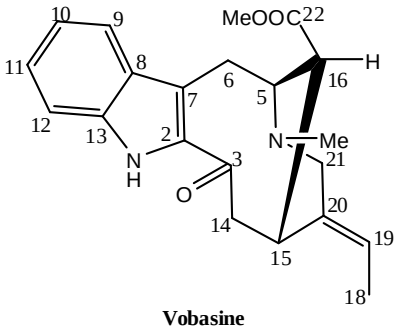
The structure of alkaloid III was deduced from analyses of the 2D NMR data, including COSY, HMQC and ^{1}H-^{13}C HMBC spectra in methanol-d_4 (Figure 3.13). The COSY and HMQC spectra revealed connectivities of three partial structures *a*-*c*; *a* (H-9 to H-10), *b* (H-6 to H-14) and *c* (H-19 to H-18) (Figure 3.13).

The ^{1}H-^{13}C HMBC cross-peaks of H-9 to C-7, H-6 to C-8 and H-14 to C-2 revealed the connectivity of partial structure *a* with *b* and the attachment of partial structure *b* to the indole moiety, while the ^{1}H-^{13}C HMBC cross-peaks of H-19 to C-15 and C-21 revealed the connectivity of partial structure *b, c* and the connection of isolated aminomethylene to partial structure *c*. In addition, the ^{1}H-^{13}C HMBC cross-peak of H-21 to C-5 established the connection of the isolated aminomethylene with partial structure *b*. The presence of a methyl carboxylate moiety at C-16 and carboxylate moiety at C-3 were analysed by ^{1}H-^{13}C HMBC correlation as shown in Figure 3.13.

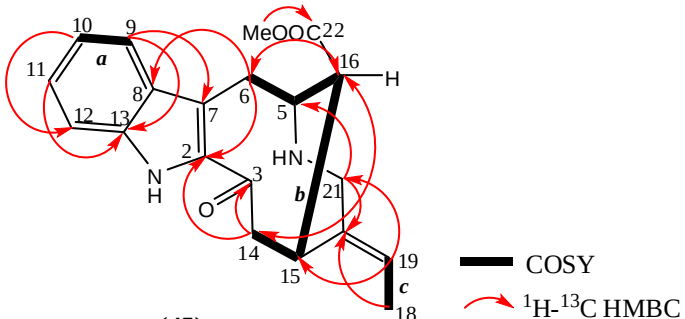
Table 3.3: ^{1}H and ^{13}C-NMR Data for Alkaloid III with comparison of the literature ^{13}C value of Vobasine[103].

Position	Alkaloid III		Vobasine
	δ_H	δ_C	δ_C
2	-	134.3	134.0
3	-	190.5	190.2
5	4.14 (br-s, 1H, overlapped with H_{21})	51.0	57.2
6	3.63 (m, 1H) 3.50 (m, 1H)	25.7	20.3
7	-	120.4	120.3
8	-	128.6	128.4
9	7.70 (d, 1H J=8.2 Hz)	121.2	120.8
10	7.13 (br-s, 1H)	120.7	120.3
11	7.32 (br-s, 2H)	127.0	126.6
12		112.0	111.8
13	-	136.6	136.4
14	3.34 (t, 1H, J=13.3 Hz) 2.74 (dd, 1H, J=7.3, 13.3 Hz)	43.4	43.0
15	3.82 (m, 1H)	31.3	30.4
16	2.65 (overlapped with 22-OMe)	49.6	46.7

18	1.67 (d, 3H, J=6.7 Hz)	12.4	12.3
19	5.41 (br-q, 1H, J=6.7 Hz)	120.4	120.8
20	-	137.8	135.8
21	4.11 (d, 1H, overlapped with H_5) 3.23 (d, 1H, J=15.1 Hz)	43.8	51.8
22	-	171.2	171.2
22-OMe	2.62 (s, 3H, overlapped with H_{16})	50.7	50.4
N(1)H	9.12, br-s	-	-
N(4)H/Me	8.76, br-s	-	42.3



Vobasine



(45)

Figure 3.13: COSY and selected ^{1}H-^{13}C HMBC correlation of alkaloid III.

Therefore, the COSY and ^{1}H-^{13}C HMBC correlation as shown in Figure 3.13 together with comparison of ^{13}C data literature value of vobasine[103] confirmed the identity of alkaloid III as the known corynanthean indole alkaloid, perivine **(45)**.

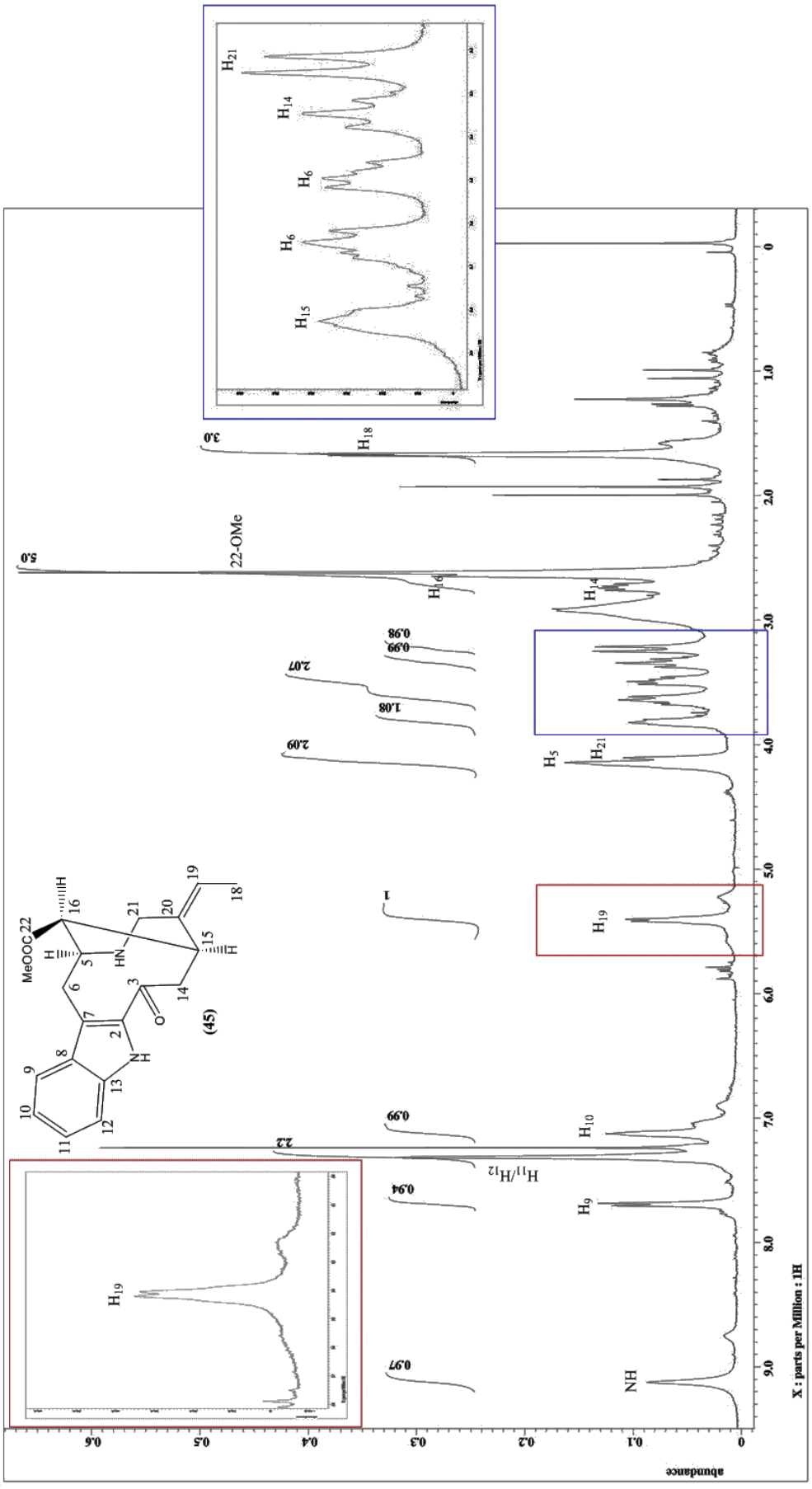


Figure 3.14: ^{1}H-NMR spectrum of alkaloid III

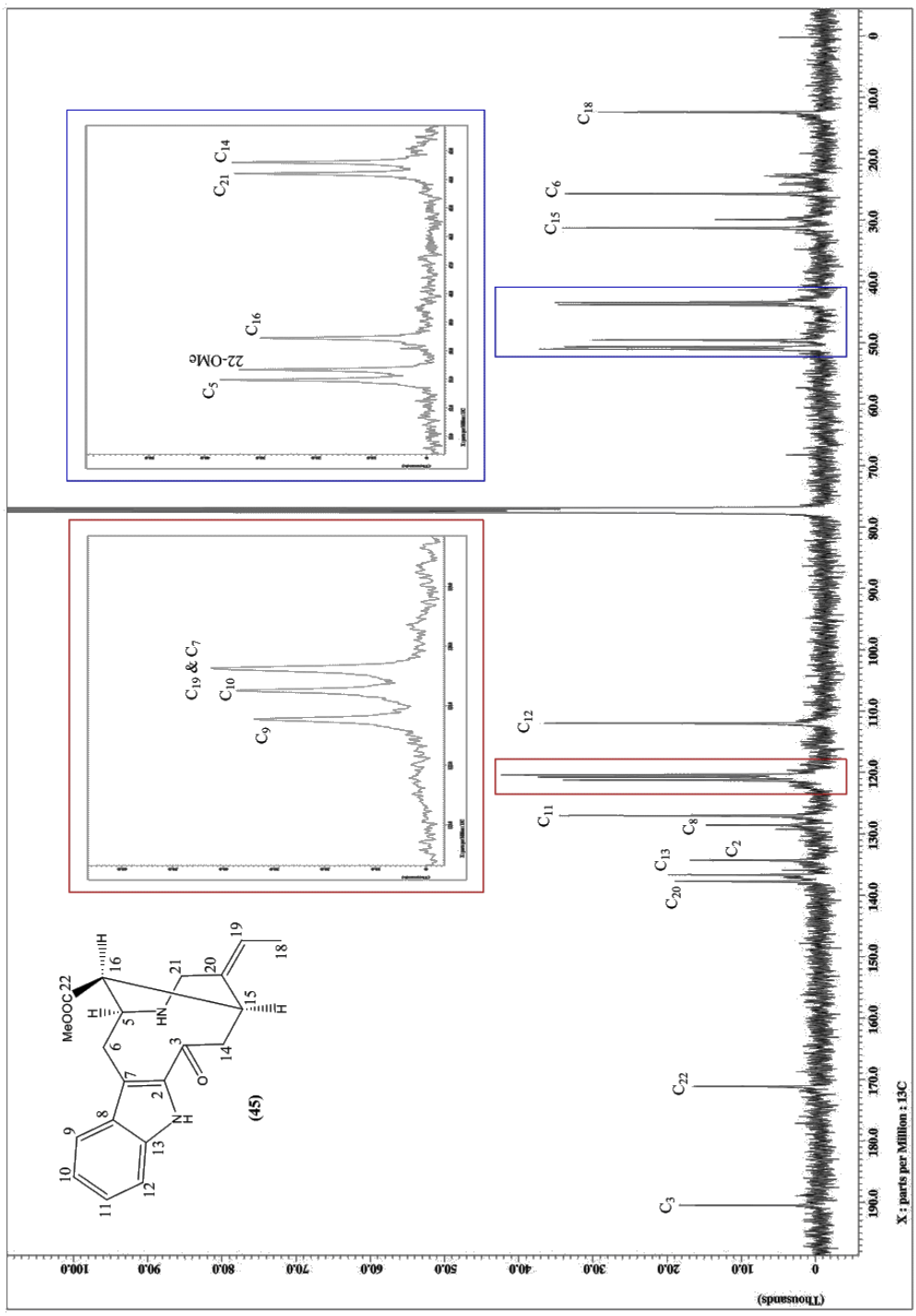


Figure 3.15: ^{13}C-NMR spectrum of alkaloid III

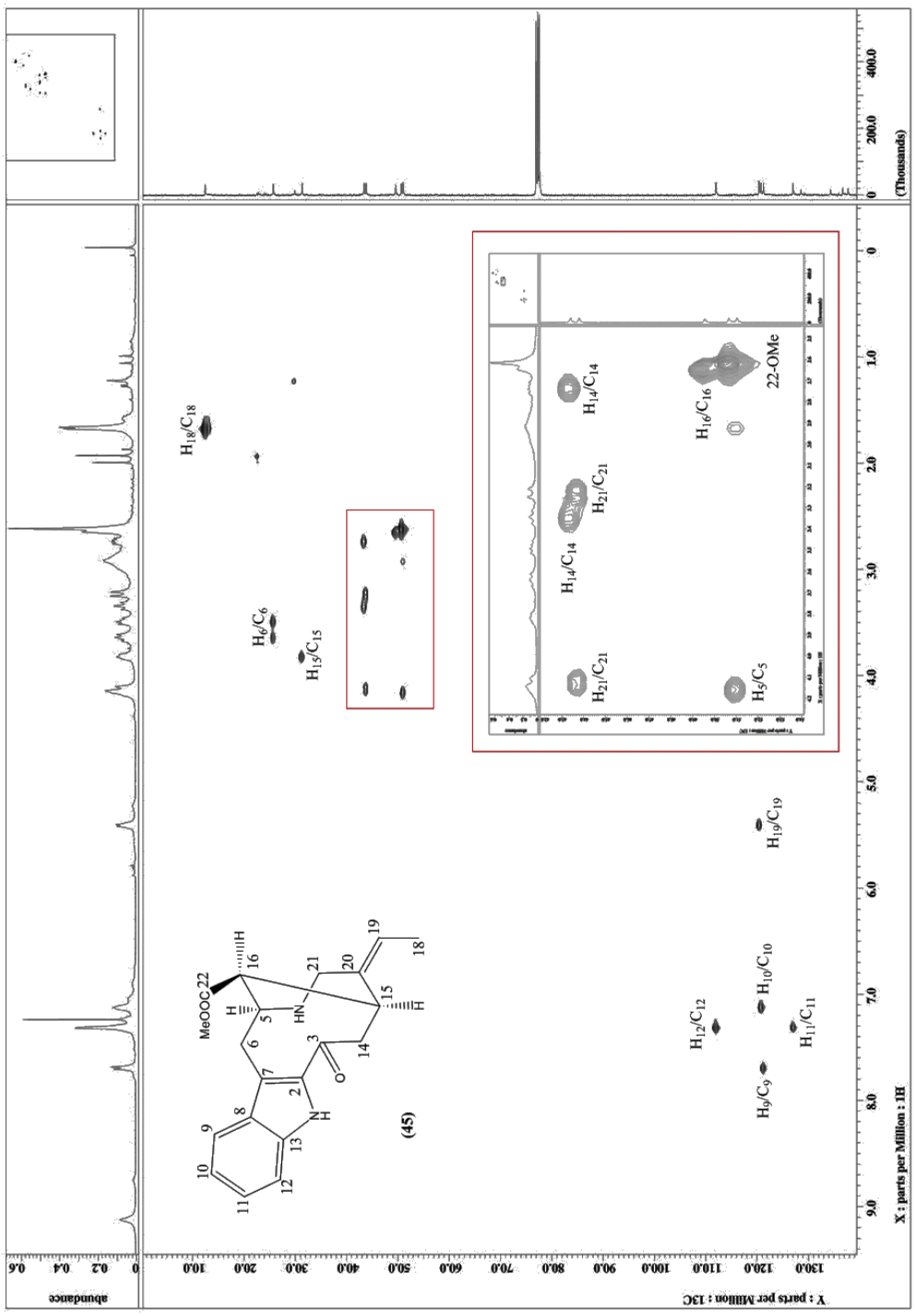


Figure 3.16: HMQC-NMR spectrum of alkaloid III

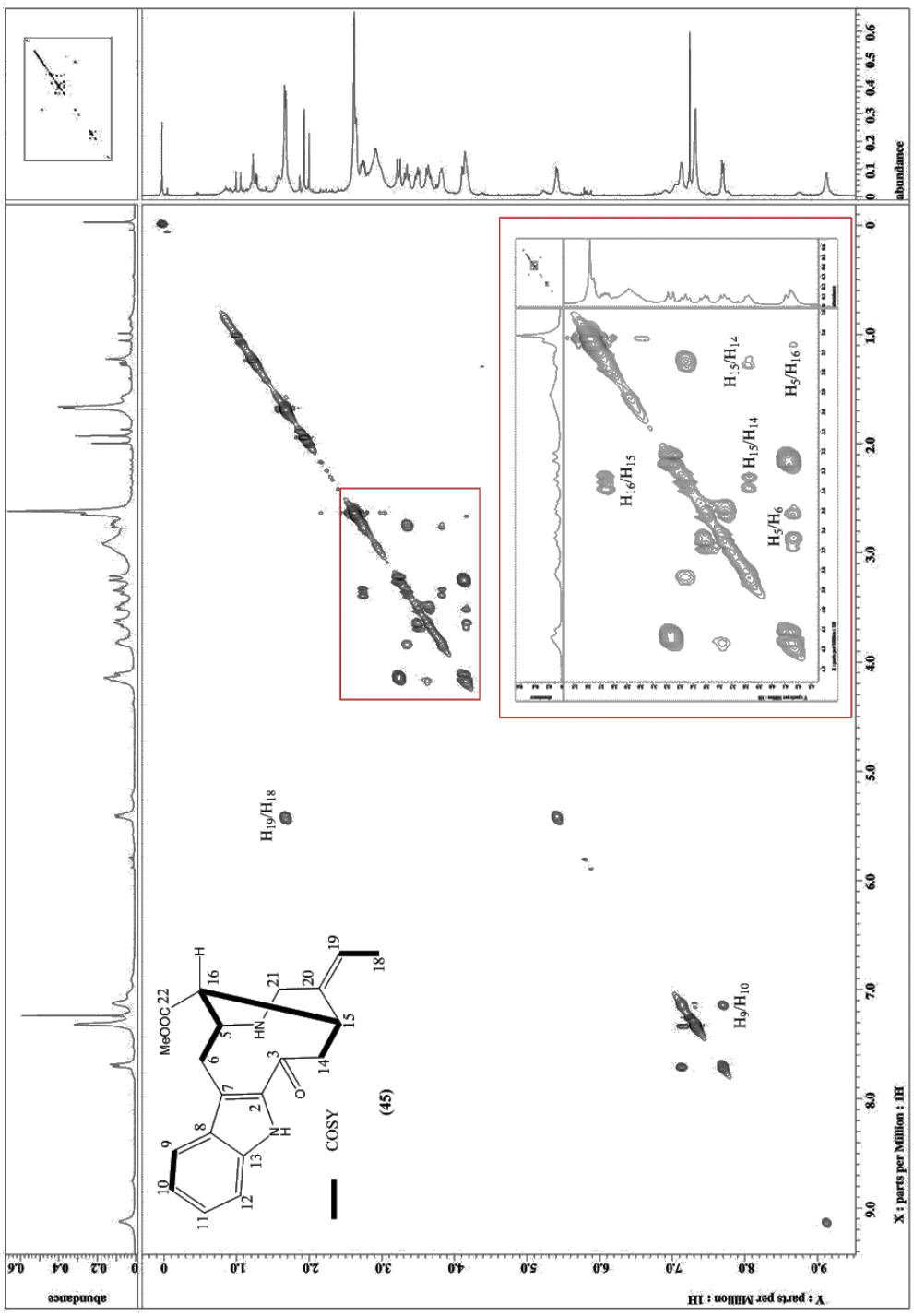


Figure 3.17: COSY-NMR spectrum of alkaloid III

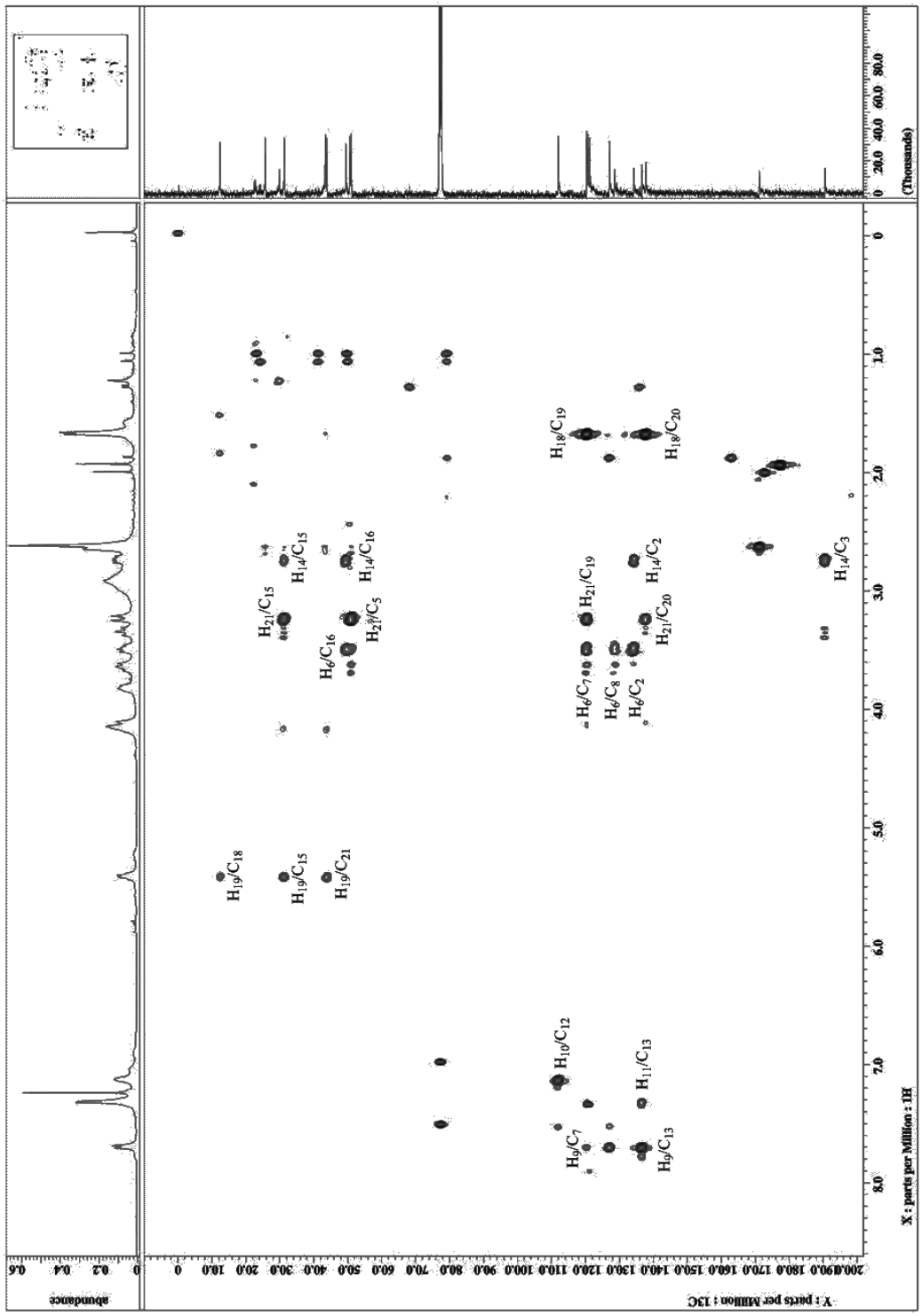
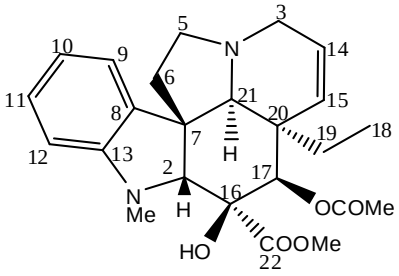


Figure 3.18: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid III

3.5 Alkaloid IV: Vindorosine (65)



(65)

Alkaloid IV isolated as yellowish brown amorphous solid. The ESIMS showed a pseudomolecular ion peak at *m/z* 427 $(M+H)^+$ and the molecular formula $C_{24}H_{30}N_2O_5$ was established by ESI-TOFMS [*m/z* 427.2290 $(M+H)^+$, Δ +5.5 mmu]. Alkaloid IV showed difference of 30 *m/z* in mass as compared to vindoline **(4)**. Thus, suggesting alkaloid IV as derivative of vindoline **(4)** lacking of a methoxy functionality. IR absorptions implied the presence of hydroxyl (3422 cm^{-1}) and ester carbonyl ester (1741 cm^{-1}) functionalities.[97] Alkaloid IV showed UV absorption of λ_{max}^{MeOH} $(\log \epsilon)$: 203 (4.16), 252 (3.83) and 308 (3.50) nm suggests a dihydroindole moiety.[98]

The ^{13}C NMR data of alkaloid IV revealed the presence of three sp^3 methine, four sp^3 methylene, four methyl, six sp^2 methine and seven quaternary carbons. The ^1H and ^{13}C NMR data of alkaloid IV show close similarity with vindoline **(4)** as shown in Table 3.4. The significant difference is the addition of an aromatic proton (δ_{H11} 7.12, t, 1H, J = 7.7 Hz) with the disappearance of 11-OMe signals as compared to those of vindoline **(4)** in ^1H NMR spectrum. Therefore, C-11 is not substituted. The downfield shift of C-8 (δ_C 133.1), C-10 (δ_C 119.3), C-12 (δ_C 109.6) and upfield shift of C-11 (δ_C 129.0) were observed as compared to those of vindoline **(4)** in the ^{13}C NMR spectrum.

Alkaloid IV identity was confirmed as known plumeran indole alkaloid, vindorosine **(65)**, through detail analysis of 2D NMR data, including COSY, HMQC

and ^{1}H-^{13}C HMBC spectra with comparison of ^{1}H and ^{13}C data of vindoline **(4)** literature value.[99; 100]

Table 3.4: ^{1}H & ^{13}C NMR Data for Alkaloid IV compared with literature of vindoline **(4)**. [99; 100]

Position	Alkaloid IV		Vindoline **(4)**	
	δ_H	δ_C	δ_H	δ_C
2	3.71 (s, 1H)	82.9	3.75	83.2
3	3.49 (α, dd, $1H_a$, J = 4.1, 16.3 Hz) 2.81 (β, d, $1H_b$, J = 16.3 Hz)	51.2	3.40	50.9
5	3.41 (α, m, $1H_a$) 2.52 (β, m, $1H_b$)	52.4	2.1-3.0	51.9
6	2.36 (m, 2H)	44.3		43.9
7	-	52.4	-	52.6
8	-	133.1	-	124.9
9	7.00 (d, 1H, J = 7.7 Hz)	122.4	6.91	122.4
10	6.74 (t, 1H, J = 7.7 Hz)	119.3	6.30	104.5
11	7.12 (t, 1H, J = 7.7 Hz)	129.0	-	161.1
12	6.50 (d, 1H, J = 7.7 Hz)	109.6	6.08	95.6
13	-	152.6	-	153.6
14	5.81(dd, 1H, J = 4.1, 10.4 Hz)	124.1	5.88	123.9
15	5.20 (d, 1H, J = 10.4 Hz)	130.4	5.23	130.2
16	-	79.6	-	79.5
17	5.45 (α, s, $1H_a$)	76.5	5.43	76.2
18	0.43 (t, 3H, J = 7.3 Hz)	7.7	0.48	7.5
19	1.61 (α, q, $1H_a$, J = 7.3) 1.05 (β, q, $1H_b$, J = 7.3)	30.9	1.35	30.6
20	-	43.1	-	42.8
21	2.65 (s, 1H)	67.4	2.65	67.0
22	-	172.0	-	171.1
OCOMe	-	170.9	-	170.4
COMe	2.05 (s, 3H)	21.2	2.07	20.8
NMe	2.66 (s, 3H)	38.6	2.68	38.0
11-OMe	-	-	3.80	55.1
22-OMe	3.77 (s, 3H)	52.4	3.80	51.9

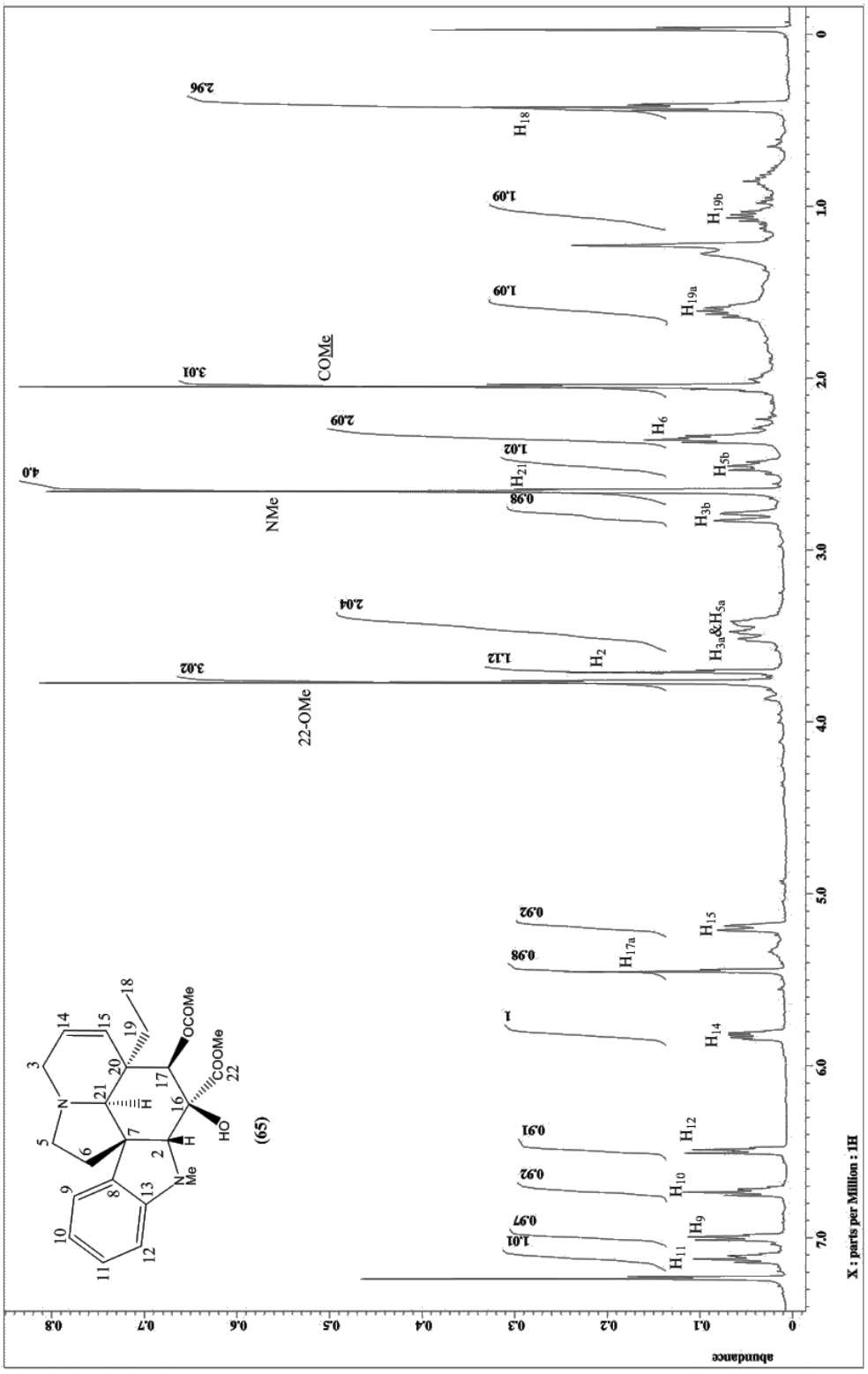


Figure 3.19: ^{1}H-NMR spectrum of alkaloid IV

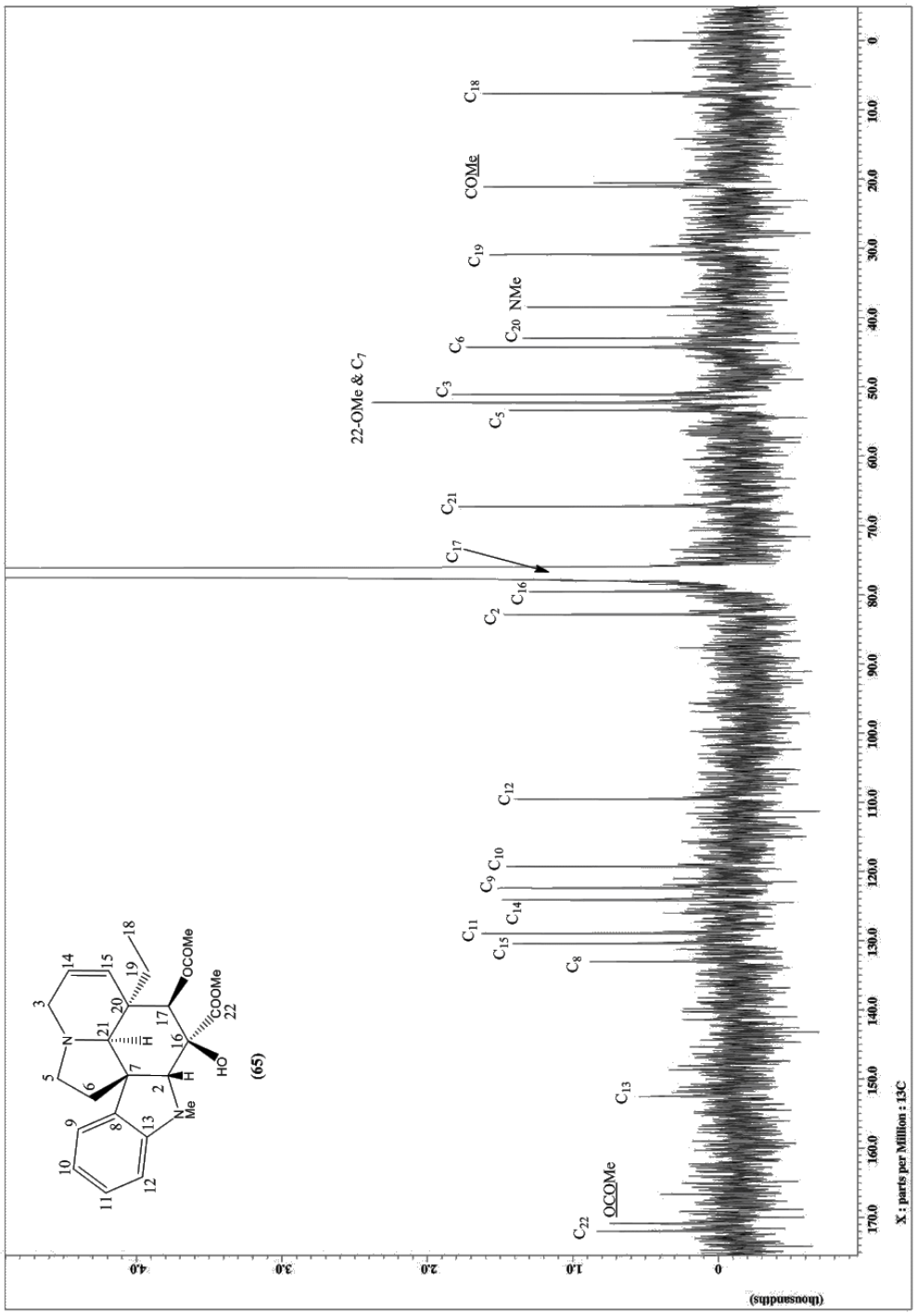
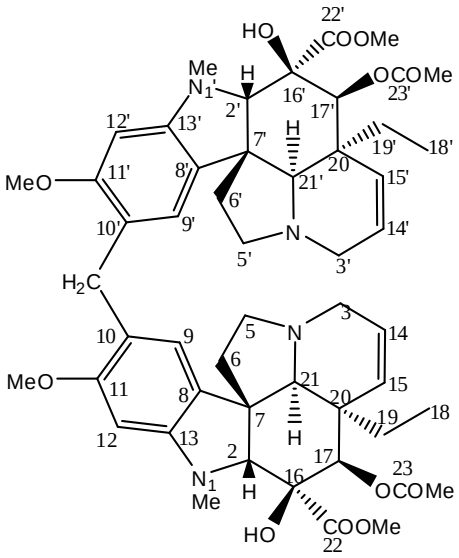


Figure 3.20: ^{13}C-NMR spectrum of alkaloid IV

3.5 Alkaloid V: Vindolicine (90)



(90)

Alkaloid V, $[\propto]_D^{22}$ -75° (*c* 1.0, MeOH) was obtained as yellowish brown amorphous solid. The ESIMS showed a pseudomolecular ion peak at *m/z* 925 $(M+H)^+$ and the molecular formula $C_{51}H_{64}N_4O_{12}$ was established by ESI-TOFMS [*m/z* 925.4665 $(M+H)^+$, Δ +6.6 mmu]. The large mass value indicated the dimeric nature of alkaloid V. The ESIMS spectrum also revealed the presence of $(M/2+H)^+$ fragment ion peak (*m/z* 463) which implied that alkaloid V to be a dimer consists of the same monomer.

IR absorptions implied the presence of hydroxyl (3432 cm^{-1}) and carbonyl ester (1741 cm^{-1}) functionalities.[97] Alkaloid V showed UV absorption of λ_{max}^{MeOH} ($\log \epsilon$): 217 (4.61), 253 (4.37) and 308 (4.03) nm reveal the presence of dihydroindole moiety.[98]

Analysis of the ^1H and ^{13}C NMR data (Table 3.5) and the HMQC spectrum of alkaloid V revealed the presence of three sp^3 methine, five sp^3 methylene, five methyl, four sp^2 methine and nine quaternary carbons. The ^1H and ^{13}C NMR spectra of alkaloid V were very similar to those of vindoline **(4)** except the addition of a methylene group (δ_H 1.21, s; δ_C 29.82) and loss of proton signal of H-10/H-10'. The ^1H-NMR spectrum of alkaloid V revealed presence of four aromatic singlets for H-9, 9' and H-12, 12' at δ_H

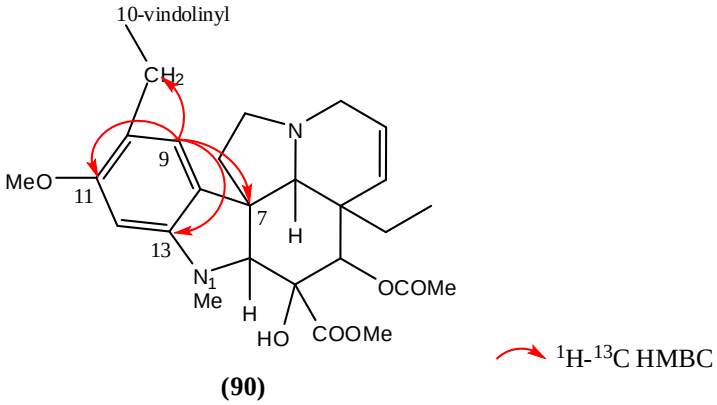
6.74 and δ_H 6.01 respectively, thus suggesting that these protons are situated *para* to each other. Therefore, indicating that C-11/C-11' and C-10/C-10' are substituted.

Table 3.5: ^{1}H and ^{13}C-NMR Data for Alkaloid V with comparison with ^{13}C-NMR data of literature vindolicine **(90)**.[71]

Position	Alkaloid V		Vindolicine **(90)**	
	δ_H	δ_C	δ_C	$\delta_{C'}$
2, 2'	3.62 (s, 1H)	83.7	83.0	
3, 3'	3.44 (dd, 1H, J=4.5, 16.3 Hz) 2.73 (d, 1H, J=16.3 Hz)	51.2	50.9	51.0
5, 5'	3.35 (ddd, 1H, J=3.6, 8.4 Hz) 2.40 (d, 1H, J=8.4 Hz)	52.4	50.8	50.6
6, 6'	2.25 (m, 2H)	43.9	42.7	41.5
7, 7'	-	53.1	52.7	52.8
8, 8'	-	123.8	123.4	
9, 9'	6.74 (s, 1H)	124.0	123.7	124.1
10, 10'	-	121.1	124.4	
11, 11'	-	158.6	159.0	
12, 12'	6.01 (s, 1H)	93.6	93.5	93.0
13, 13'	-	151.9	152.1	
14, 14'	5.78 (dd, 1H, J=4.5, 10.3 Hz)	124.2	123.9	124.4
15, 15'	5.15 (d, 1H, J=10.3 Hz)	130.7	130.2	130.0
16, 16'	-	79.7	79.3	79.5
17, 17'	5.41 (s, 1H)	76.5	75.9	76.0
18, 18'	0.31 (t, 3H, J=7.3 Hz)	7.9	7.1	7.1
19, 19'	1.54 (q, 1H, J=7.3 Hz) 1.00 (q, 1H, J=7.3 Hz)	31.0	30.5	30.3
20, 20'	-	43.1	42.5	42.4
21, 21'	2.49 (s, 1H)	67.3	67.0	
22, 22'	-	172.2	170.6	
23, 23'-Me	2.02 (s, 3H)	21.2	20.7	
N_1/N_1'-Me	2.60 (s, 3H)	39.1	38.0	37.7
11, 11'-OMe/'	3.74 (s, 6H)	55.5	55.1	
22, 22'-OMe/'		52.4	52.0	51.8
23, 23'	-	170.9	171.6	171.4
CH_2	1.21 (s, 2H)	29.8	34.0	

The structure of alkaloid V was deduced to consist of two vindoline **(4)** unit that form a methylene bridge at C-10/C-10' to be a symmetrical dimer. The HMBC spectrum showed correlation signal between CH_2 with H-9/H-9' and C-11/C11'. Thus, signifying that both monomers are connects through CH_2. The ^{1}H-^{13}C HMBC cross-

peak of H-9/H-9' to CH_2 established the connection for each vindoline **(4)** monomer through a methylene bridge.



(90)

Figure 3.21: Selected ^{1}H-^{13}C HMBC correlation of alkaloid V.

Finally, thorough analysis of 2D NMR that includes HMQC, COSY and ^{1}H-^{13}C HMBC together with comparison of the ^{13}C literature values confirmed the identity of alkaloid V as the known plumeran bisindole alkaloid, vindolicine **(90)**.

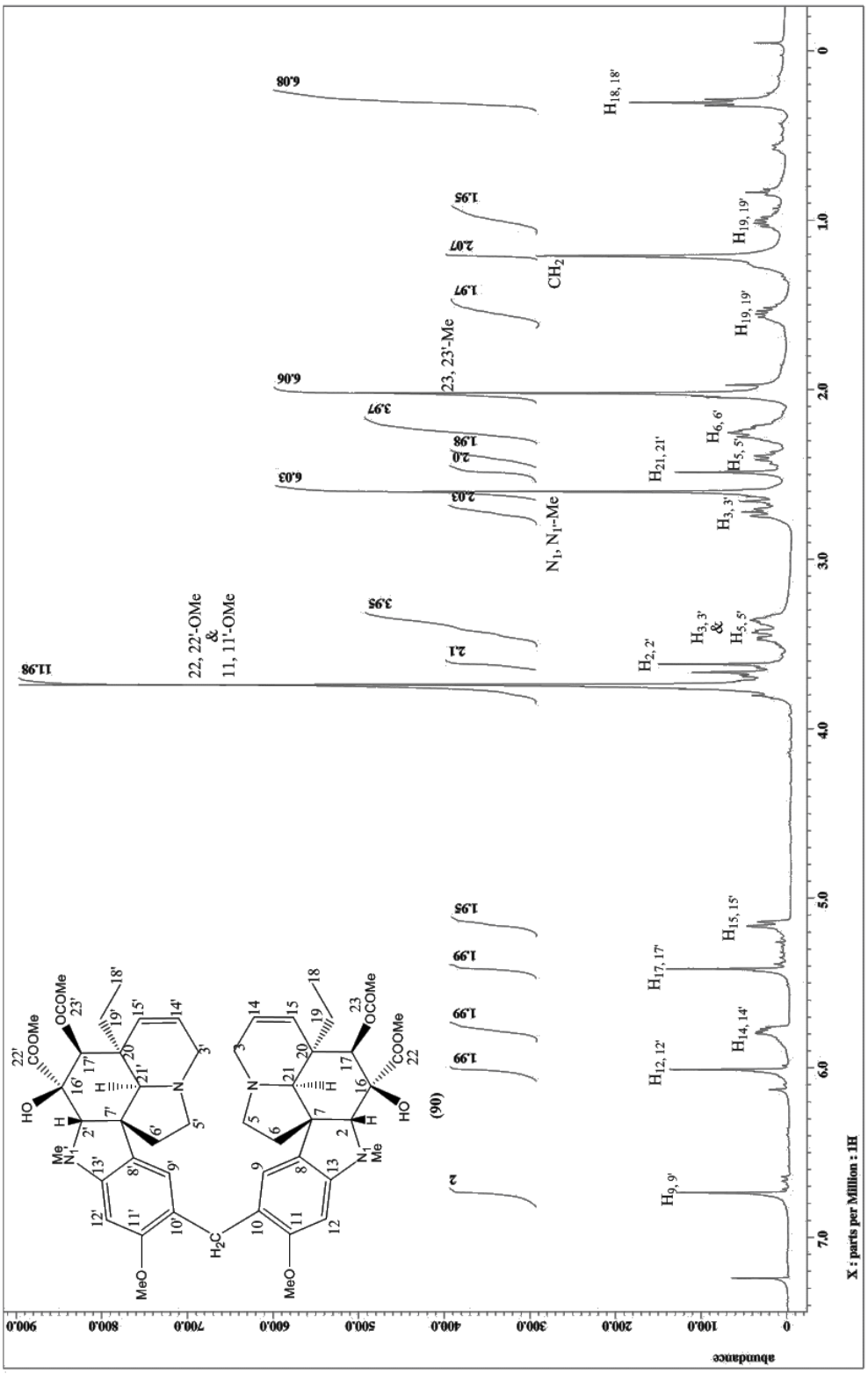


Figure 3.22: ^{1}H-NMR spectrum of alkaloid V

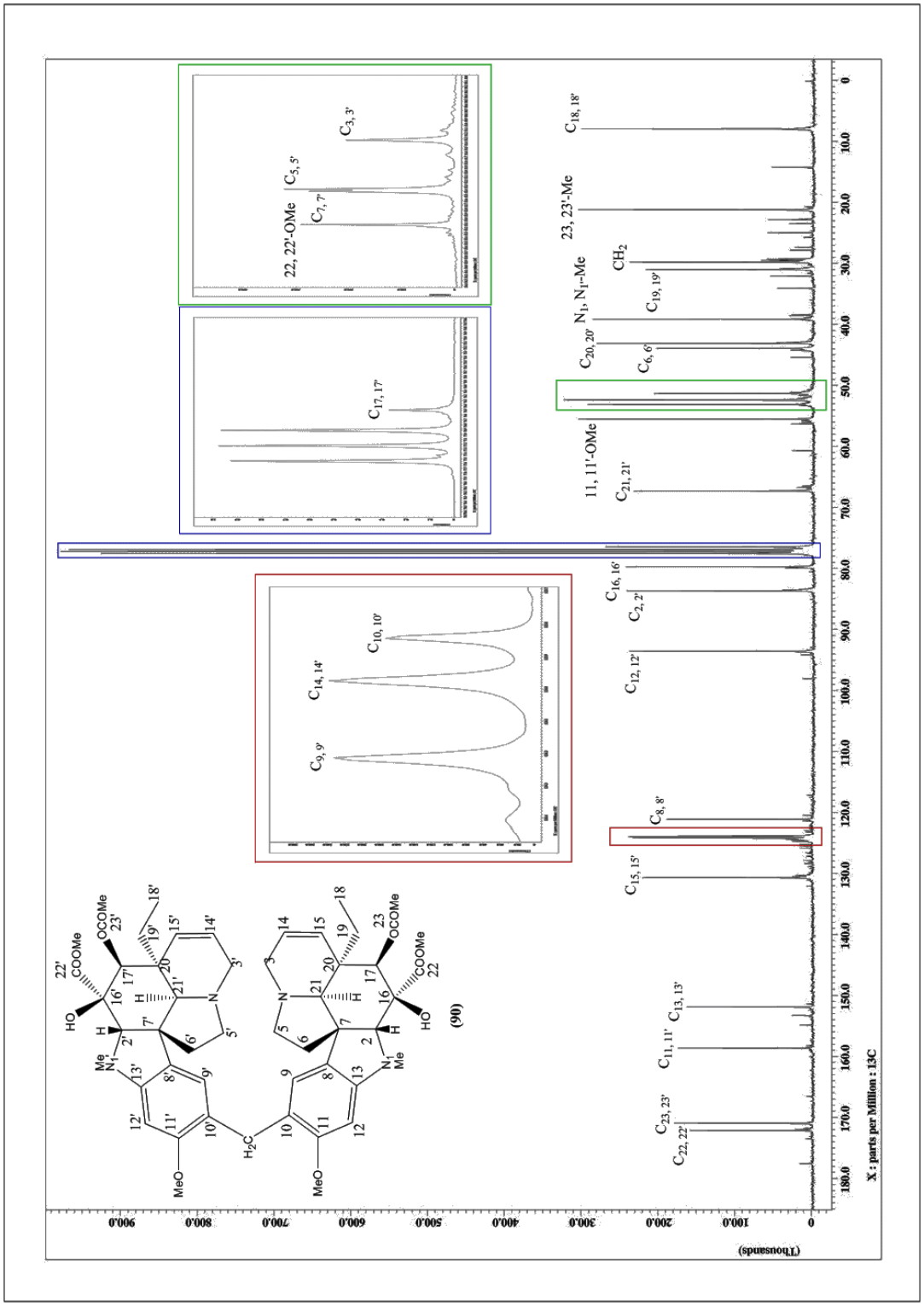


Figure 3.23: ^{13}C-NMR spectrum of alkaloid V

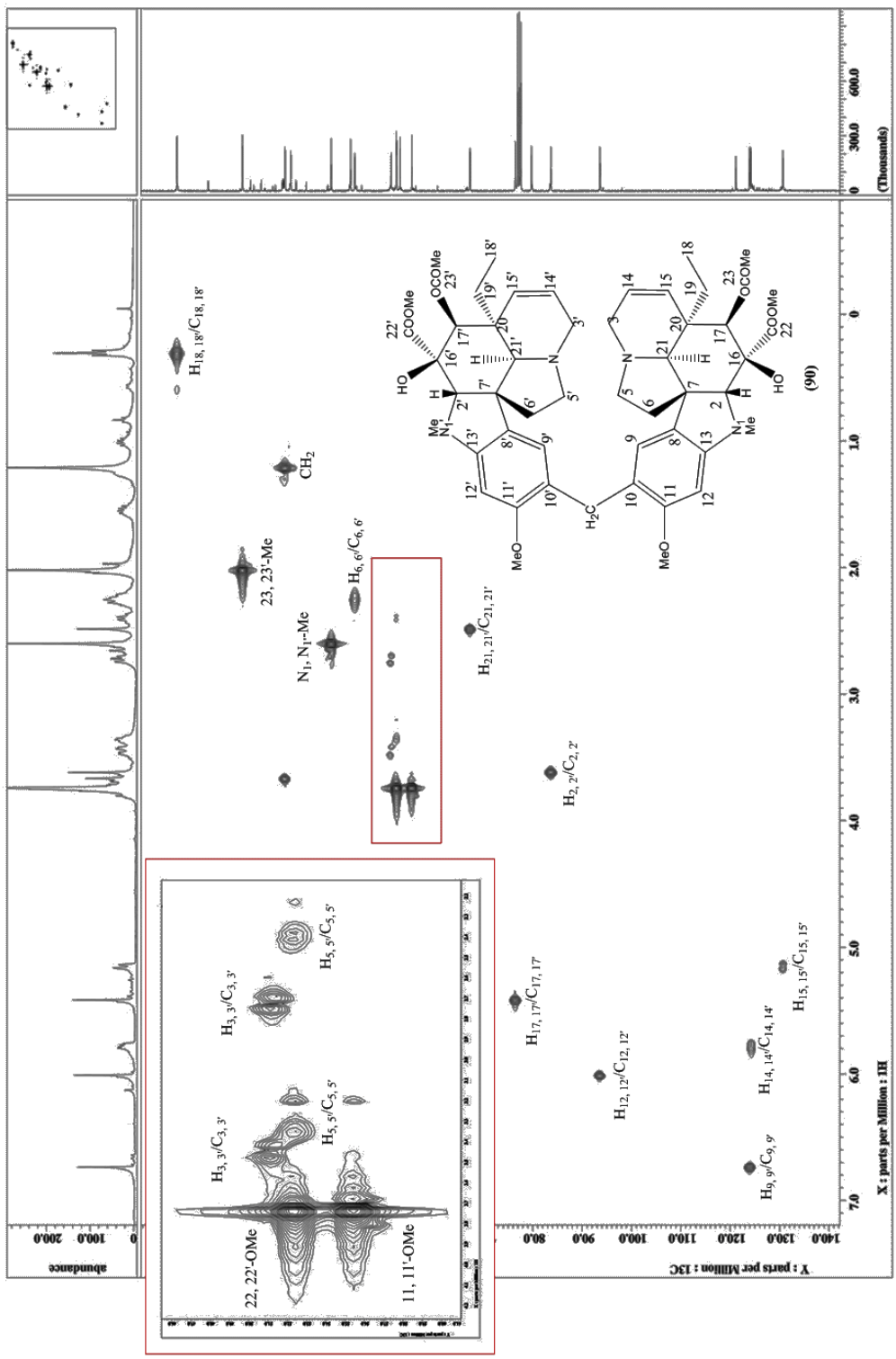


Figure 3.24: HMQC-NMR spectrum of alkaloid V

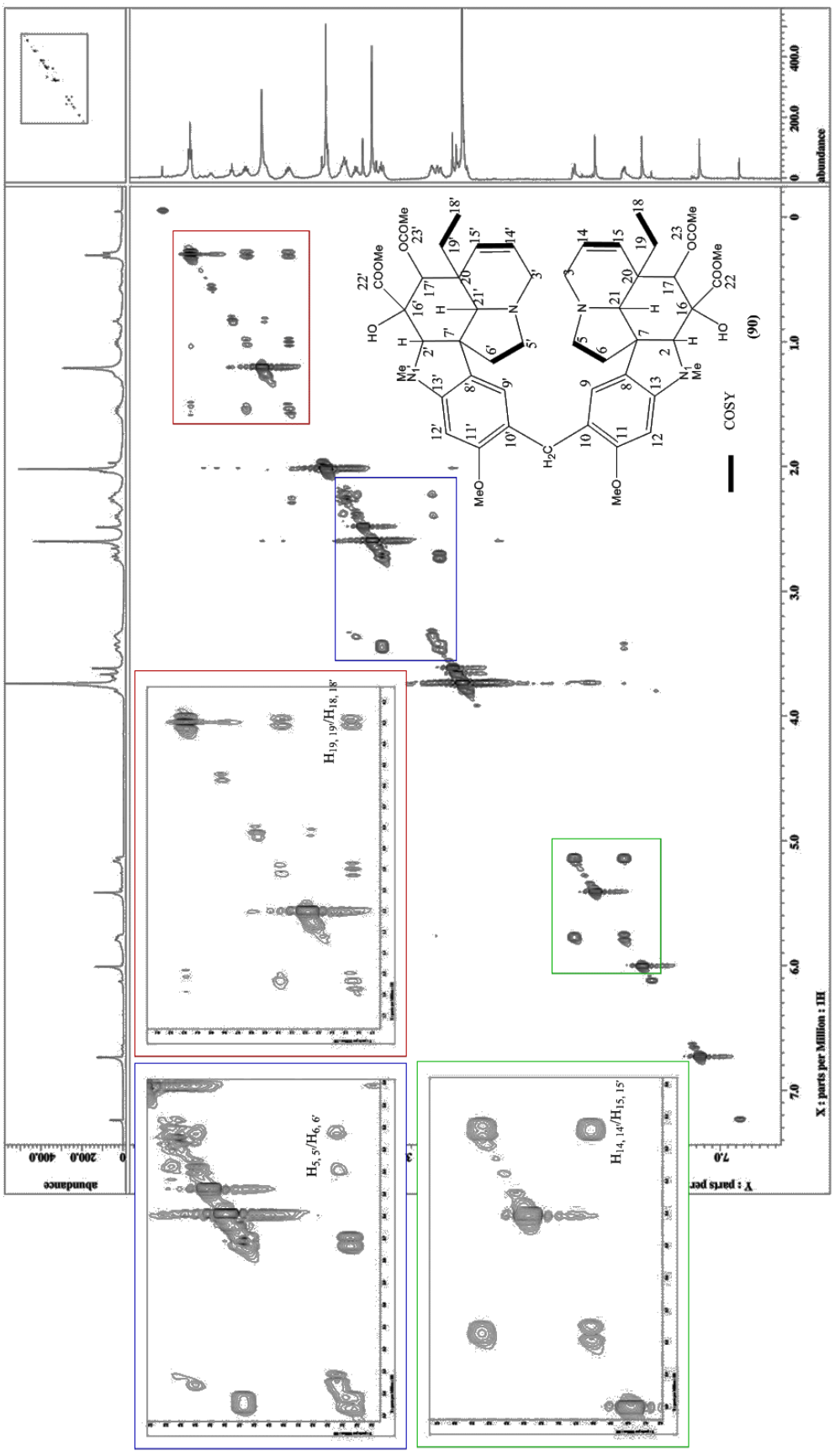


Figure 3.25: COSY-NMR spectrum of alkaloid V

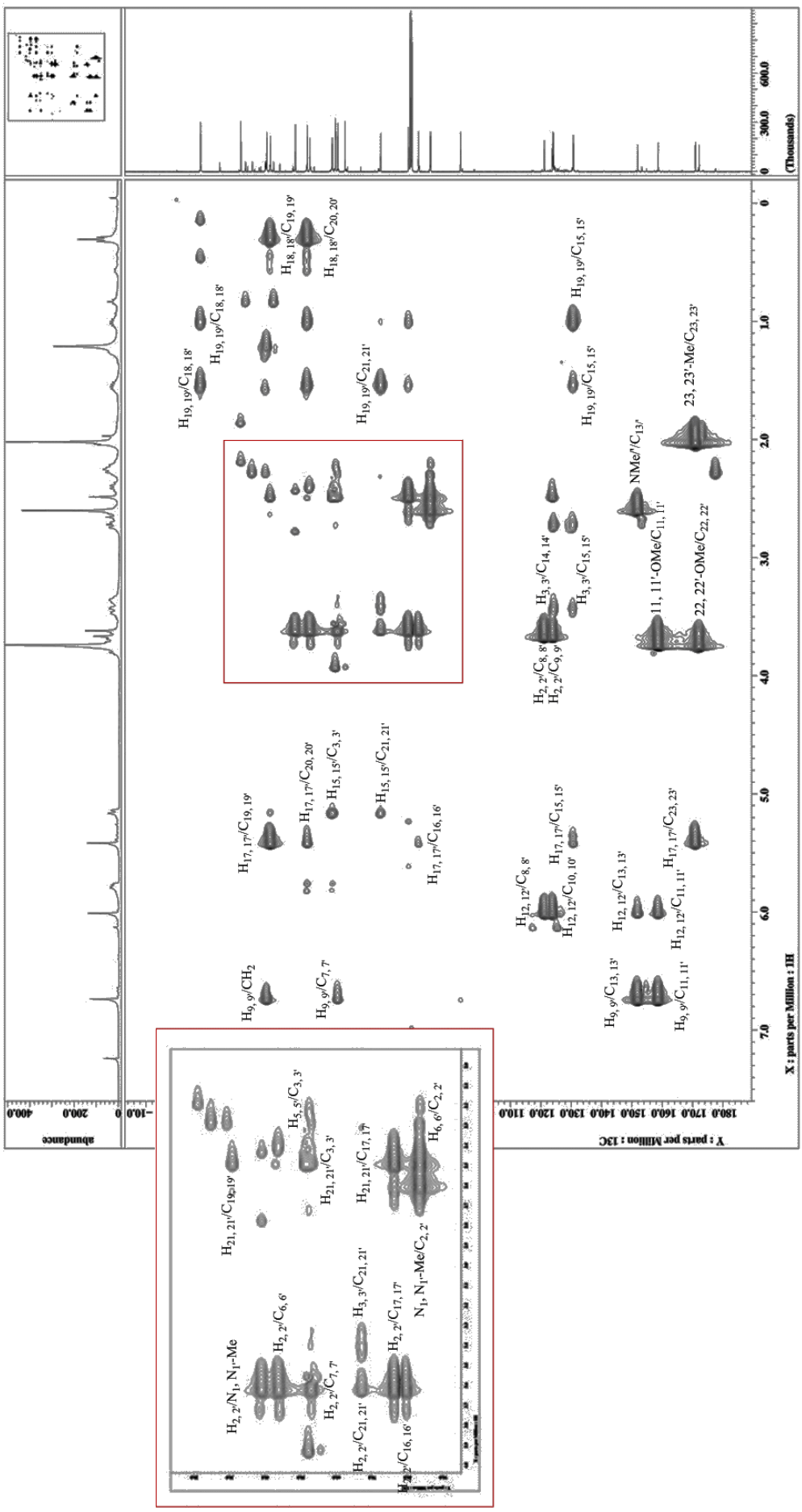
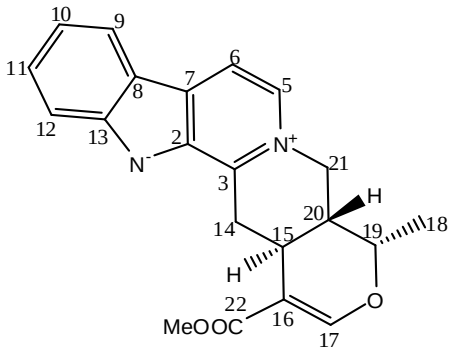


Figure 3.26: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid V

3.7 Alkaloid VI: Serpentine (104)



(104)

Alkaloid VI, $[\propto]_D^{22}$ +60° (*c* 1.0, MeOH) obtained as yellowish brown amorphous solid. ESIMS spectrum showed a pseudomolecular ion peak at *m/z* 349 $(M+H)^+$ and the molecular formula $C_{21}H_{20}N_2O_3$ was established by ESI-TOFMS [*m/z* 349.1560 $(M+H)^+$, Δ +0.6 mmu]. Alkaloid VI showed UV absorption of λ_{max}^{MeOH} $(\log \epsilon)$: 251 (4.20), 308 (4.02) and 363 (3.58) nm.

Analysis of the ^1H and ^{13}C NMR data (Table 3.6) and the HMQC spectrum of alkaloid VI revealed the presence of three sp^3 methine, two sp^3 methylene, two methyl, seven sp^2 methine and seven quaternary carbons. The presence of an ester functionality was supported by the observed quaternary signal for C-22 at δ_C 168.8 and the corresponding oxygenanted methyl resonance at δ_C 51.8. Two highly downfield carbon signals at δ_C 156.4 and δ_C 107.2 were belong to an oxygenated methine C-17 and quaternary carbons C-16 of an olefin system respectively. Another two signals for an aromatic aminomethine C-5 and amino-quaternary C-3 carbons were observed at δ_C 134.3 and δ_C 141.3 respectively.

^1H NMR spectrum of alkaloid VI showed the presence of six aromatic protons signal at δ_H 7.43 (H-10), δ_H 7.72-7.78 (H-11 & H-12), δ_H 8.33 (H-5 & H-9) and δ_H 8.47 (H-6). An oxygenated olefinic proton H-17 signal at δ_H 7.70 and a methoxy group

associated with a methyl ester function as singlet at δ_H 3.77 (22-OMe), A characteristic methyl protons, H-18 that appear as a doublet at δ_H 1.33 (J=6.6 Hz) was also observed.

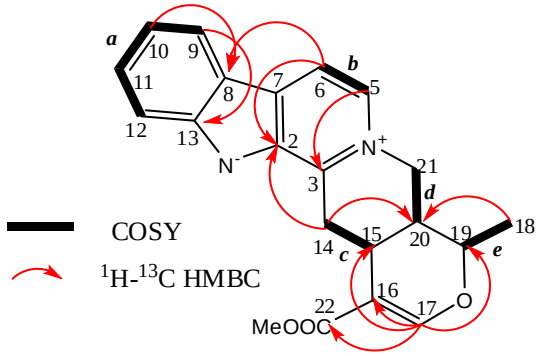
Table 3.6: ^{1}H and ^{13}C-NMR Data for Alkaloid VI compared with the literature of Serpentine **(104)**[104].

Position	Alkaloid VI		Serpentine **(104)**	
	δ_H	δ_C	δ_H	δ_C
2	-	135.8	-	135.8
3	-	141.3	-	141.2
5	8.33 (overlapped with H_9)	134.3	8.31 (d, J=6.6 Hz)	134.2
6	8.47 (d, 1H, J=6.1 Hz)	116.8	8.46 (d, J=6.6 Hz)	116.8
7	-	132.6	-	132.6
8	-	121.4	-	121.3
9	8.33 (overlapped with H_5)	124.1	8.31 (dd, J=1.0, 8.1 Hz)	124.0
10	7.43 (t, 1H, J=7.1 Hz)	123.2	7.43 (ddd, J=1.3, 6.6, 8.1 Hz)	123.2
11	7.72-7.78 (br-s, 2H)	132.9	7.76 (ddd, J= 1.0, 6.6, 8.4 Hz)	132.9
12		114.0	7.72 (dd, J=1.3, 8.4 Hz)	113.9
13	-	145.4	-	145.4
14	4.68 (dd, 1H, J= 4.9, 18.2 Hz) 3.15 (dd, 1H, J=11.5, 18.2 Hz)	31.9	4.64 (α, dd, J=4.7, 18.1 Hz) 3.13 (β, dd, J=11.6, 18.1 Hz)	31.9
15	3.06 (dt, 1H, J=4.9, 11.5 Hz)	26.0	3.04 (dddd, J=1.4, 4.7, 11.5 Hz)	26.0
16	-	107.2	-	107.2
17	7.70 (s, 1H)	156.4	7.71 (d, J=1.4 Hz)	156.3
18	1.33 (d, 3H, J=6.6 Hz)	14.3	1.34 (d, J=6.8 Hz)	14.2
19	4.75 (dd, 2H, J=4.1, 6.6 Hz)	73.0	4.75 (qd, J=6.8, 4.2 Hz)	72.9
20	2.68 (t, 1H, J=11.5 Hz)	38.5	2.67 (dddd, J=4.1, 4.2, 11.5, 13.0 Hz)	38.5
21	4.87 (overlapped with CD_3OD) 4.61 (dd, 1H, J= 13.3, 13.0 Hz)	57.6	4.88 (β, dd, J=13.3, 4.1 Hz) 4.59 (α, dd, J=13.3, 13.0 Hz)	57.6
22	-	168.4	-	168.4
22-OMe	3.77 (s, 3H)	51.8	3.82 (s)	51.8

The structure of alkaloid VI was deduced from analyses of the 2D NMR data, including COSY, HMQC and ^{1}H-^{13}C HMBC spectra in methanol-d_4 (Figure 3.27). The COSY and HMQC spectra revealed connectivities of five partial structures *a*-*e*; *a* (H-9

to H-12), *b* (H-5 to H-6), *c* (H-14 to H-15), *d* (H-20 to H-21) and *e* (H-18 to H-19) (Figure 3.27).

The ^{1}H-^{13}C HMBC cross-peaks of H-10 to C-8 and H-6 to C-8 and C-2 revealed the connection of partial structure *a*, *b* and attachment of partial structure *b* to indole moiety, while ^{1}H-^{13}C HMBC cross-peaks H-14 to C-20, C-2, H-18 to C-20 revealed the connection of partial structure *c*, *d*, *e* and attachment of partial structure *c* to indole moiety. The presence of a methyl carboxylate moiety at C-16 and an isolated sp^{2} methine at C-17 were deduced by ^{1}H-^{13}C HMBC correlation between H-17 with C-22 (Figure 3.27).



(104)

Figure 3.27: COSY and selected ^{1}H-^{13}C HMBC correlation of alkaloid VI

Therefore, the identity of alkaloid VI was confirmed as the known corynanthean indole alkaloids, serpentine **(104)** by thorough analysis together with comparison of ^{1}H and ^{13}C NMR data of literature.[104] (Table 3.6)

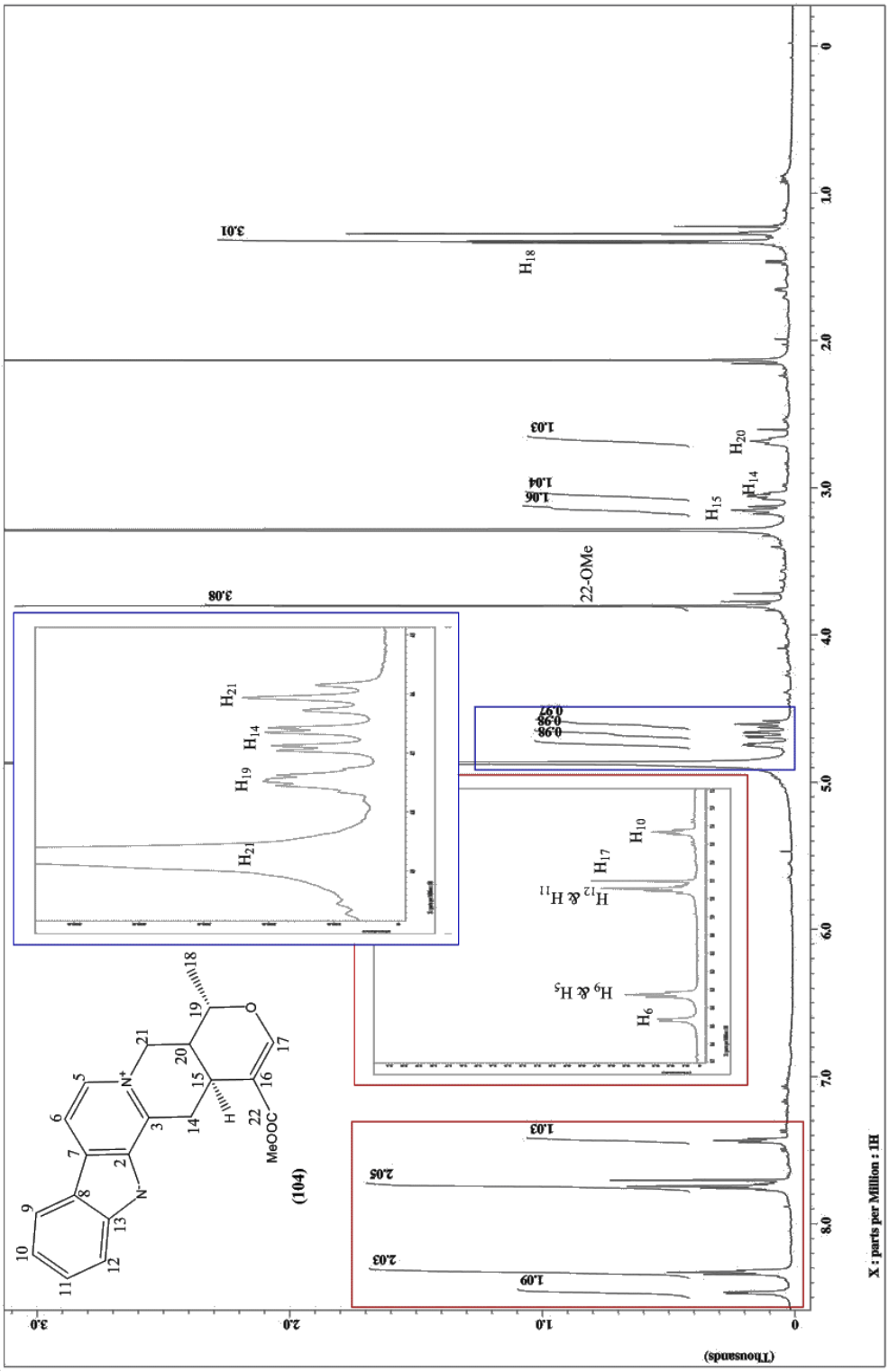


Figure 3.28: ^{1}H-NMR spectrum of alkaloid VI

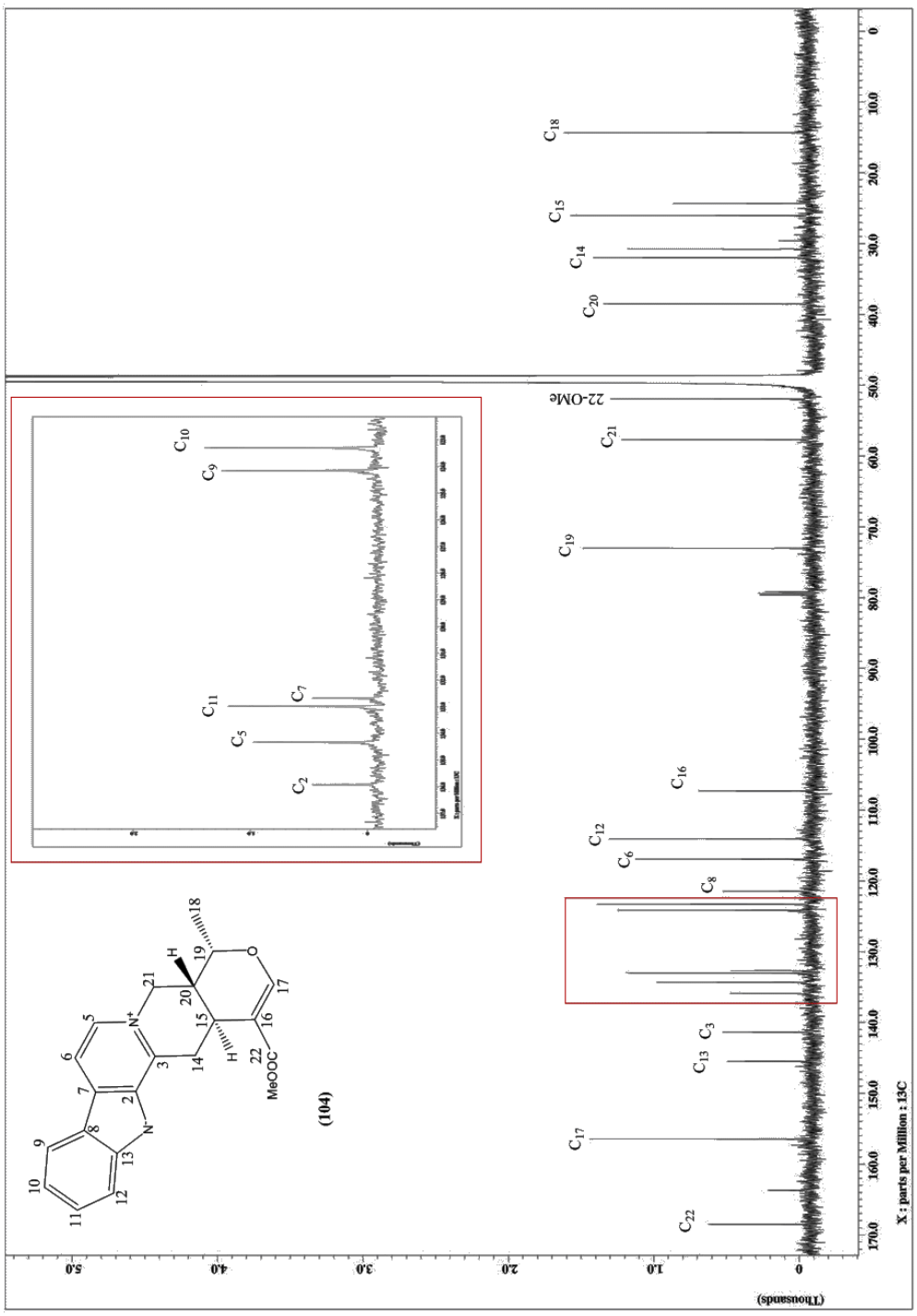


Figure 3.29: ^{13}C-NMR spectrum of alkaloid VI

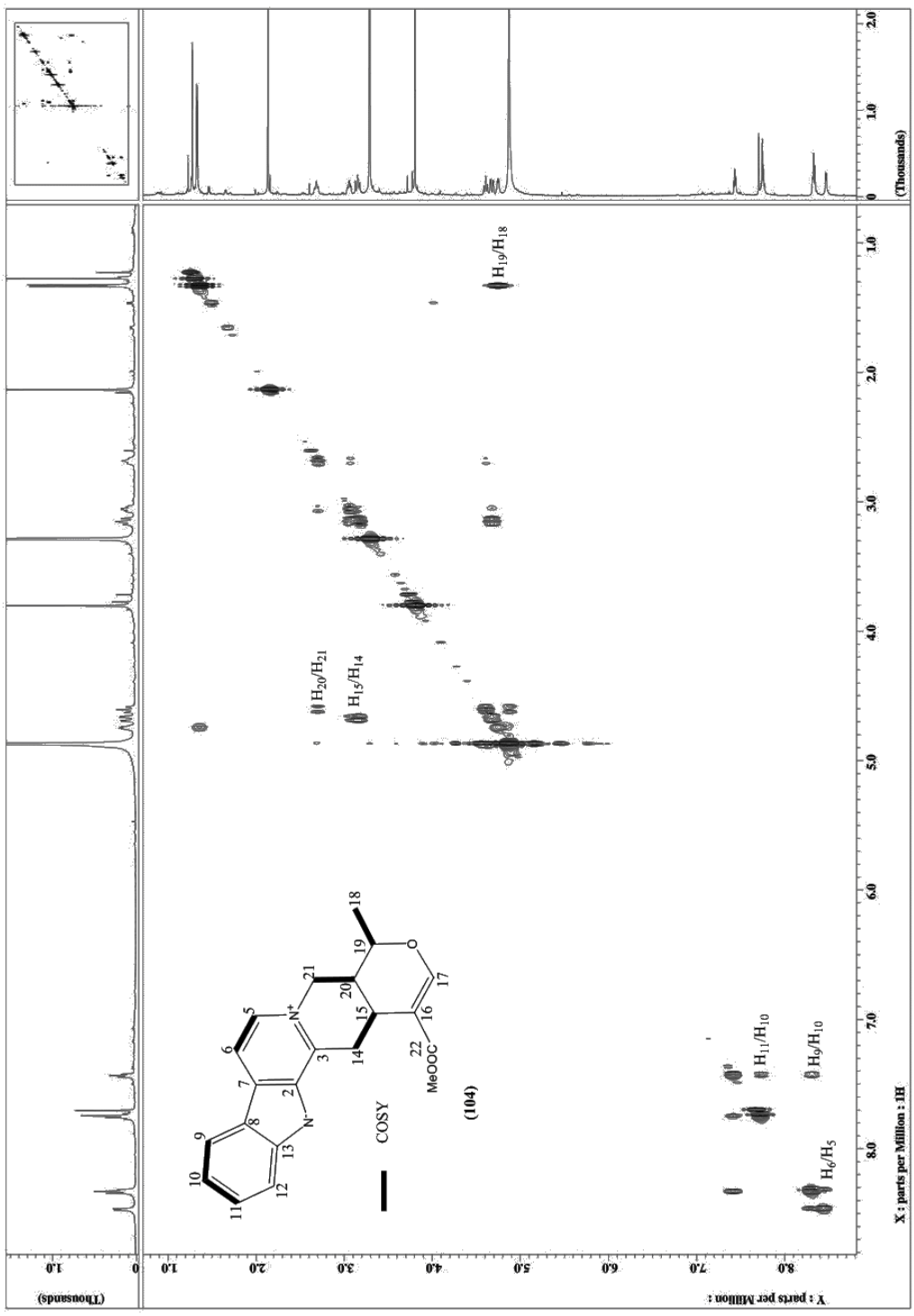


Figure 3.30: COSY-NMR spectrum of alkaloid VI

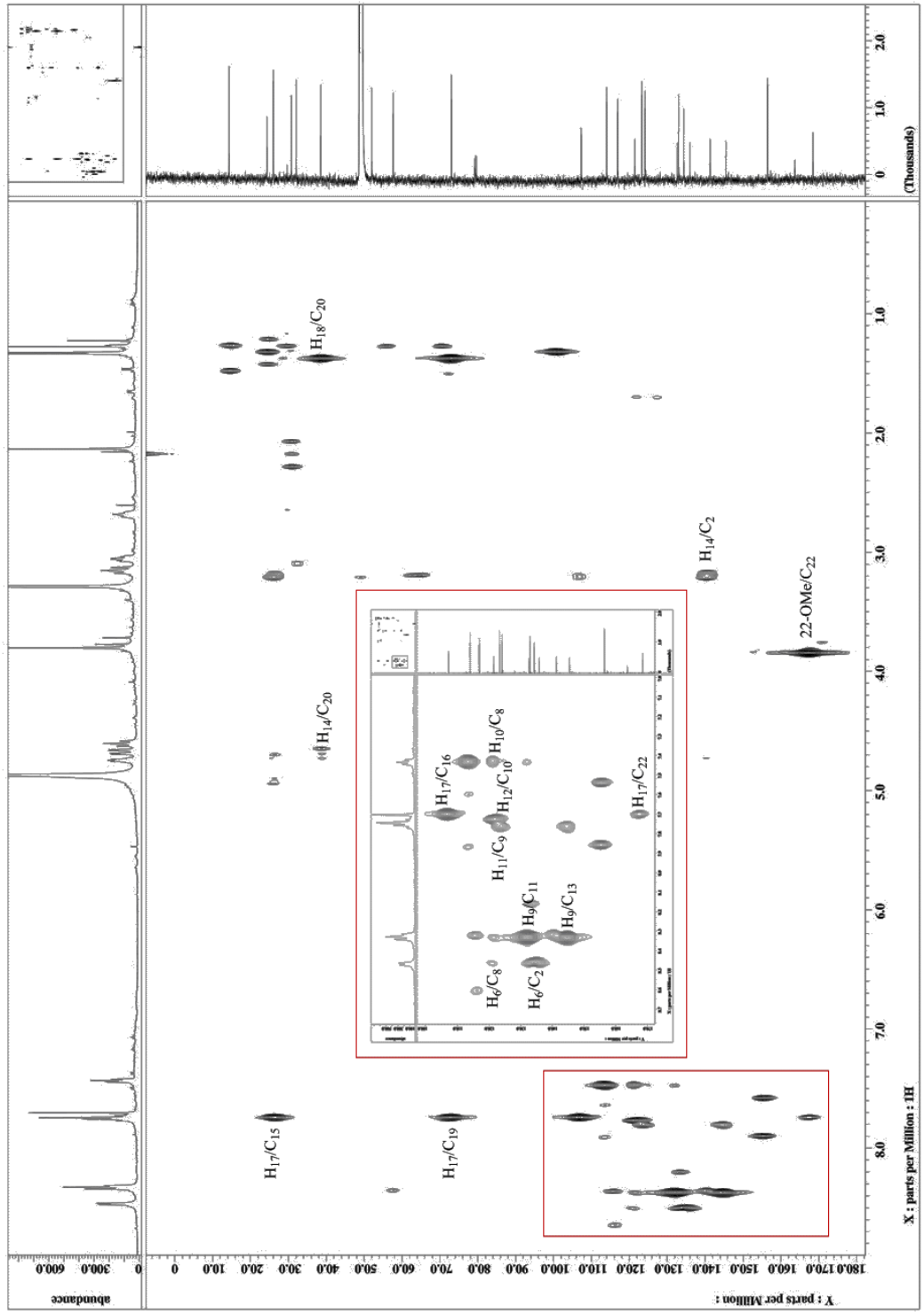
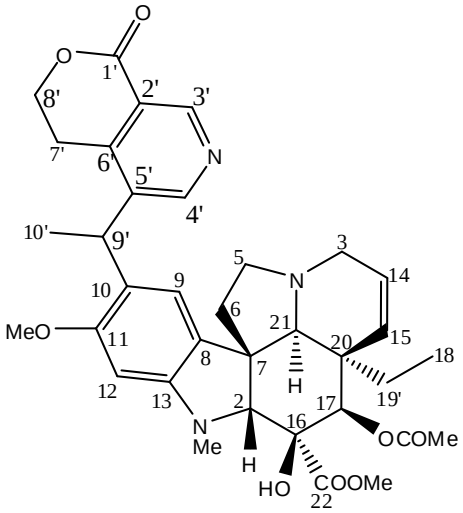


Figure 3.31: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid VI

3.8 Alkaloid VII: Vindogentianine (136)



(136)

Alkaloid VII**,** $[\propto]_D^{22}$ -134° (*c* 1.0, MeOH) was isolated as yellowish brown amorphous solid. ESIMS spectrum showed a pseudomolecular ion peak at *m/z* 632 $(M+H)^+$. The molecular formula $C_{35}H_{41}N_3O_8$ was established by ESI-TOFMS [*m/z* 632.3060 ($M+H^+$), Δ +8.9 mmu] and [*m/z* 676.2897 ($M+HCOO^-$), Δ +2.7 mmu]. IR absorptions implied the presence of hydroxyl (3458 cm^{-1}) and ester carbonyl (1741 cm^{-1}) functionalities.[97] Alkaloid VII showed UV maxima of $\lambda_{max}^{MeOH}(\log \epsilon)$: 216 (4.46), 259 (4.11) and 310 (4.01) nm thus suggesting the presence of dihydroindole moiety.[98]

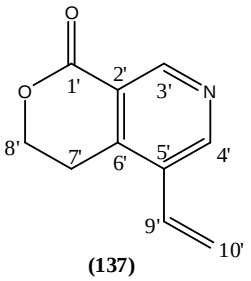
Analysis of the ^1H and ^{13}C NMR data (Table 3.7) and the HMQC spectrum of alkaloid VII revealed the presence of four sp^3 methine, six sp^3 methylene, six methyl, six sp^2 methine and thirteen quaternary carbons. Examination on the ^1H and ^{13}C NMR spectra of alkaloid VII revealed the characteristic feature of vindoline **(4)**. Ethyl moiety was exhibited in ^1H and ^{13}C NMR at δ_H 1.01 & 1.56, δ_C 30.8 and δ_H 0.49, δ_C 7.8 for methylene at C-19 and methyl at C-18 respectively. Two olefinic *cis* proton H-14 and H-15 were observed at δ_H 5.84 and δ_H 5.20 respectively. Two strong methoxy singlets at δ_H 3.74 and δ_H 3.72 that belong to 22-OMe and 11-OMe respectively. The presence of an acetoxyl functionality was indicated by the resonance at δ_C 171.0 with the corresponding acetyl resonance at δ_C 21.2. The ^1H NMR of alkaloid VII revealed a

disubstituted indole aromatic system with only two singlet proton signals for H-9 and H-12 at δ_H 6.57 and δ_H 6.00 respectively.

The vindoline **(4)** skeleton occupied twenty five carbons, whereas alkaloid VII has thirty five carbons. Therefore, the other subunit must consist of ten carbons; one sp^3 methine, two sp^3 methylene, one methyl, two sp^2 methine and four quaternary carbons. The ^{13}C NMR showed the C-1' carbonyl signal at δ_C 164.3 with the corresponding ethyl resonance at δ_C 66.6 (C-8') and δ_C 23.9 (C-7'). Two highly downfield signals at δ_C 149.5 (C-3') and δ_C 152.2 (C-4') are due to two aromatic aminomethine.

The other hydrogen atoms have six degree of unsaturation suggested the presence of six membered lactone fused together with a pyridine ring. The structure of the ten carbons subunit was deduced from analyses of the 2D NMR data, including COSY, HMQC and ^1H-^{13}C HMBC spectra (Figure 3.32). The COSY and HMQC spectra revealed connectivities of 2 partial structures consisted of $-OCH_2CH_2-$ and $-CHCH_3$ in the ten carbons subunit.

Beside the proton signals resembling vindoline **(4)** subunit in the ^1H-NMR spectrum of alkaloid VII, two highly downfield aromatic protons were observed at δ_H 9.06 (H-3') and δ_H 8.58 (H-4') confirmed the presence of pyridine system. In addition, one oxymethylene and methine signals overlap at δ_H 4.48 (H-8' & H-9'), one methylene (δ_H 2.93, H-7') and one methyl (δ_H 1.48, H-10') protons were observed.



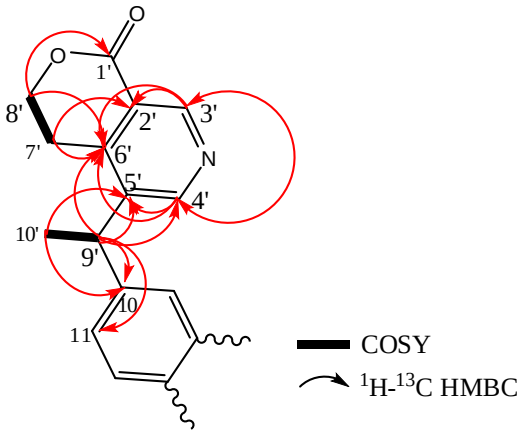
(137)

Table 3.7: ^{1}H and ^{13}C-NMR Data for Alkaloid VII compared with ^{1}H literature of vindoline **(4)**[99-101] and gentianine **(137)**[105].

Position	Alkaloid VII		Vindoline **(4)**	Gentianine **(137)**
	δ_H	δ_C	δ_H	δ_H
2	3.68 (s, 1H)	83.4	3.75	-
3	3.44 (α, dd, $1H_a$, J=4.5, 16.3 Hz) 2.83 (β, d, $1H_b$, J=16.3 Hz)	51.0	3.40	-
5	3.36 (α, ddd, $1H_a$, J=4.4, 9.2, 13.8 Hz) 2.49 (β, dd, $1H_b$, J=6.9, 10.6 Hz)	51.4	2.1-3.0	-
6	2.21 (ddd, 1H, J=2.3, 6.9, 13.8 Hz) 2.01 (m, 1H, overlap with H_{COMe})	44.2		-
7	-	53.2	-	-
8	-	124.1	-	-
9	6.57 (s, 1H)	120.6	6.91	-
10	-	122.8	6.30	-
11	-	157.6	-	-
12	6.00 (s, 1H)	93.4	6.08	-
13	-	152.4	-	-
14	5.84 (dd, 1H, J=4.5, 10.1 Hz)	124.6	5.88	-
15	5.20 (d, 1H, J=10.1 Hz)	130.4	5.23	-
16	-	79.8	-	-
17	5.34 (α, s, $1H_a$)	76.5	5.43	-
18	0.49 (t, 3H, J=7.3 Hz)	7.8	0.48	-
19	1.56 (α, q, $1H_a$, J=7.3 Hz) 1.01 (β, q, $1H_b$, J=7.3 Hz)	30.8	1.35	-
20	-	42.9	-	-
21	2.64 (s, 1H)	66.4	2.65	-
22	-	171.9	-	-
COMe	2.03 (s, 3H)	21.2	2.07	-
NMe	2.65 (s, 3H)	38.7	2.68	-
11-OMe	3.74 (s, 3H)	55.7	3.80	-
22-OMe	3.72 (s, 3H)	52.4	3.80	-
OCOMe	-	171.0	-	-
1’	-	164.3	-	-
2’	-	138.6	-	-
3’	9.06 (s, 1H)	149.4	-	9.06
4’	8.58 (s, 1H)	152.2	-	8.84
5’	-	121.1	-	-
6’	-	146.5	-	-

7’	2.92 (m, 2H, overlapped with H_3)	23.9	-	3.00
8’	4.48 (m, 2H, overlapped with $H_{9'}$)	66.6	-	4.54
9’	4.48 (m, 1H, overlapped with $H_{8'}$)	31.7	-	5.77, 5.98 and 7.08
10’	1.48 (d, 3H, J=7.3 Hz)	21.0	-	

The ^{1}H-^{13}C HMBC cross-peaks of H-10’ to C-10 and H-9’ to C-11 confirmed that both the gentianine **(137)** and vindoline **(4)** moieties were connected through the C-9’/C-10 bridge. Pyridine ring was trisubstituted with the lactone ring and a methylated methane carbon. This hypothesis was proven by the ^{1}H-^{13}C HMBC correlation between H-7’/C-2’, H-8’/C-1’, H-8’/C-6’ and H-9’/C-5’.



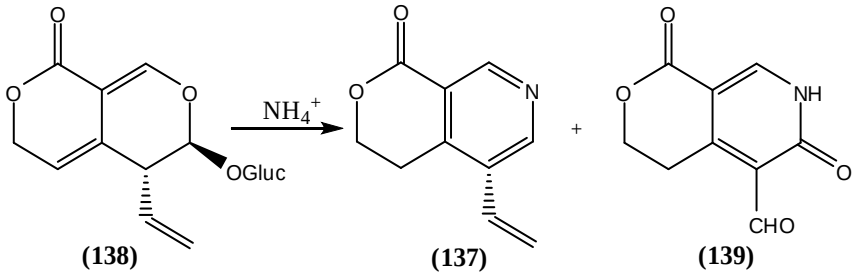
(136)

Figure 3.32: COSY and selected ^{1}H-^{13}C HMBC correlation of alkaloid VII.

Therefore, the identity of alkaloid VII as the new plumeran indole alkaloids with substitution of gentianine subunit at C-10 named as vindogentianine **(136),** was confirmed by thorough analysis of 2D NMR that include HMQC, COSY and ^{1}H-^{13}C HMBC together with comparison of ^{1}H NMR data of vindoline **(4)**[99] and gentianine **(137)**[105] literature value.

Vindogentianine **(136)** could be formed from the known plumeran alkaloid, vindoline **(4)** and gentianine **(137)**. Through literature, it was found that gentianine **(137)** was an artefact form from degradation of gentiopicroside **(138)**, a secoiridoid

glycoside.[106; 107] Gentiopicroside **(138)** is chemically unstable, it can be transformed into gentianine **(137)** and gentinal **(139)** in the presence of ammonium (Figure 3.33).[105; 108] Therefore, the existence of vindogentianine **(136)** was believed to be an artefact.



(138) **(137)** **(139)**

Figure 3.33: Condensation of gentiopicroside **(138)** with ammonium

Gentiopicroside **(138)** commonly existed in gentianceous plants especially in the genera of *Gentiana* and *Swertia* (Gentianaceae family).[108] Loganic acid **(140)**, the iridoid glycoside that responsible for the formation of monoterpenoid indole alkaloids in Apocynaceae plants was also found to be the precursor for the formation of gentiopicroside **(138)** in *Swertia* plants. Loganic acid **(141)** firstly undergoes hydroxylation to loganin **(142)** before ring cleavage to have an aldehyde moiety. *Trans* esterification with the aldehyde moiety will afford a lactol reminiscent like in sweroside **(143)**. Lastly, dehydrogenation of sweroside **(144)** will give gentiopicroside **(138)** (Figure 3.34).[106]

Gentianine **(137)** is ubiquitous in plants from the family Gentianaceae, especially in the genera of *Gentiana* and *Swertia*. [108; 109] It has been reported to occur in *Alstonia* and *Hunteria* of Apocynaceae family.[110-113] However, this is the first report of the occurrence of an alkaloid linked to gentianine **(137)** moiety.

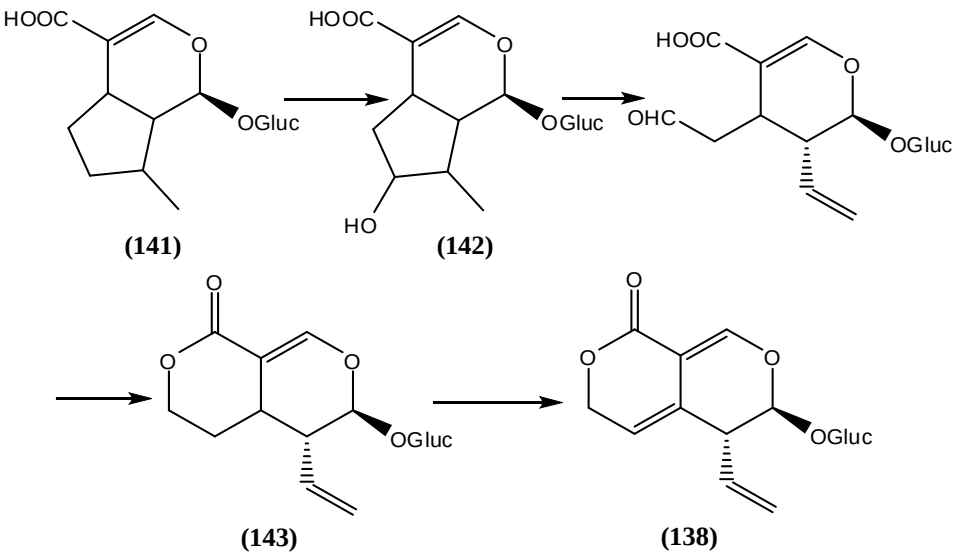


Figure 3.34: Biosynthesis of gentiopicroside **(138)** from loganic acid **(141)** in *Swertia* plants. [106]

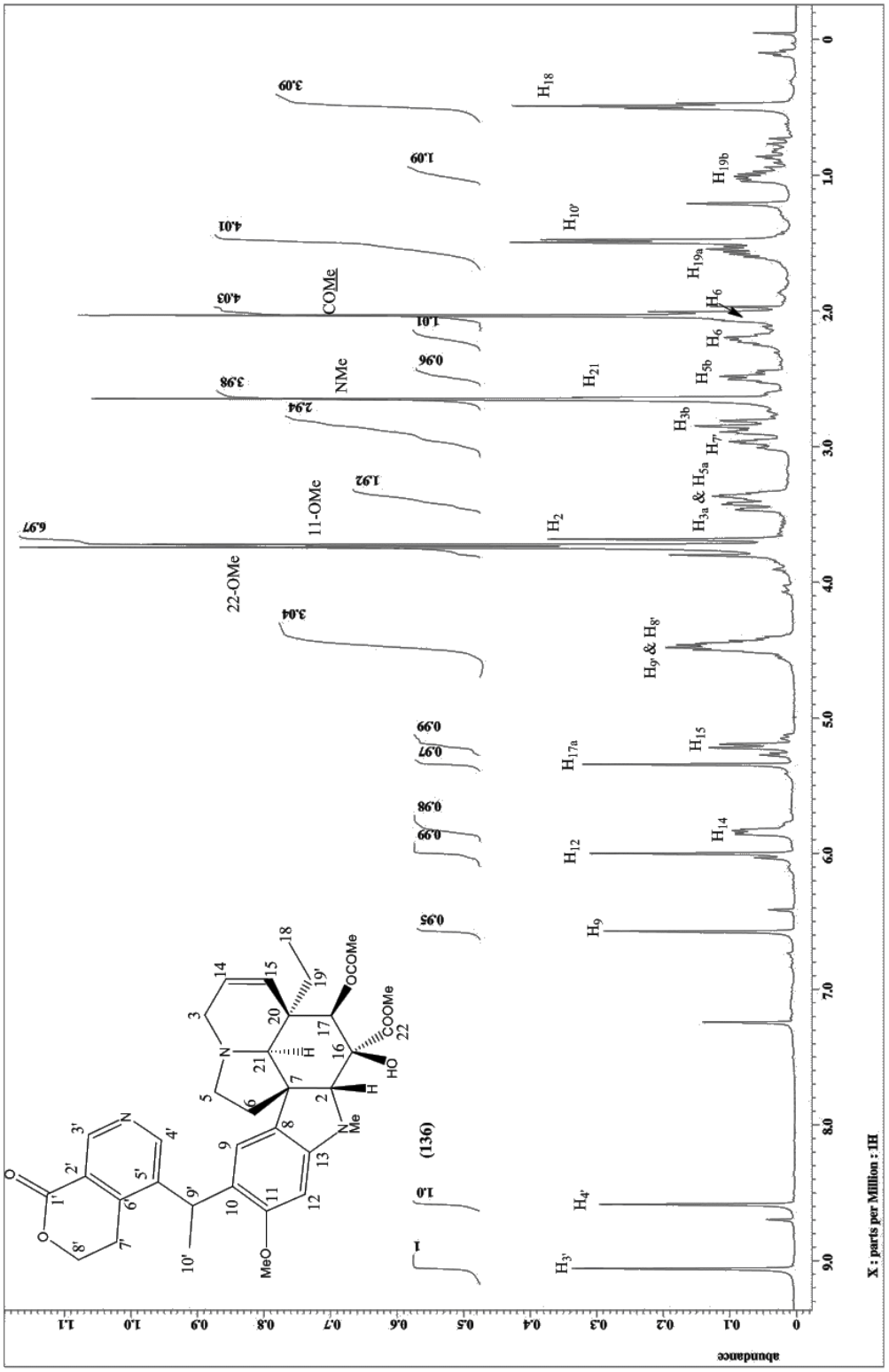


Figure 3.35: ^{1}H-NMR spectrum of alkaloid VII

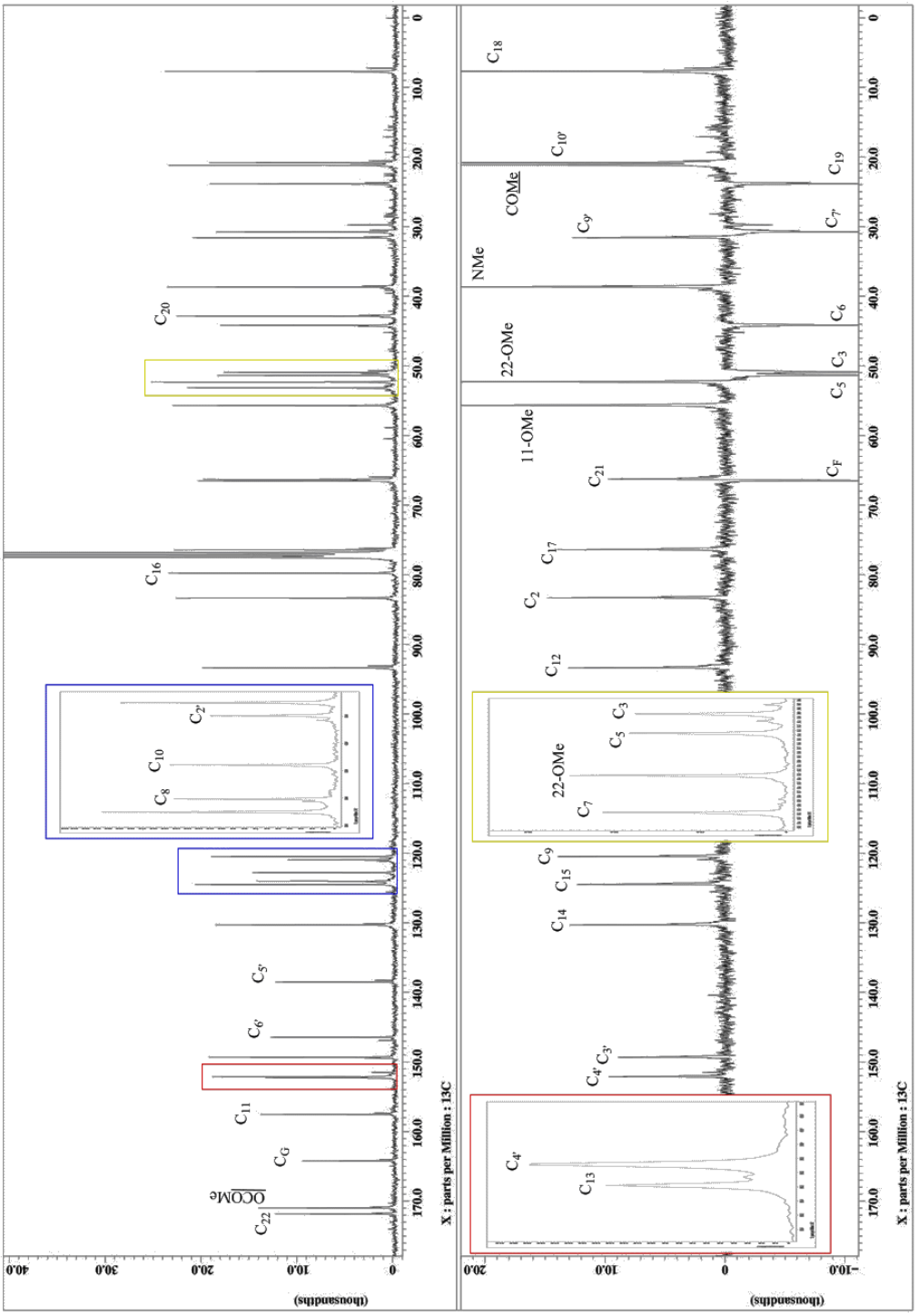


Figure 3.36: ^{13}C and DEPT NMR spectrum of alkaloid VII

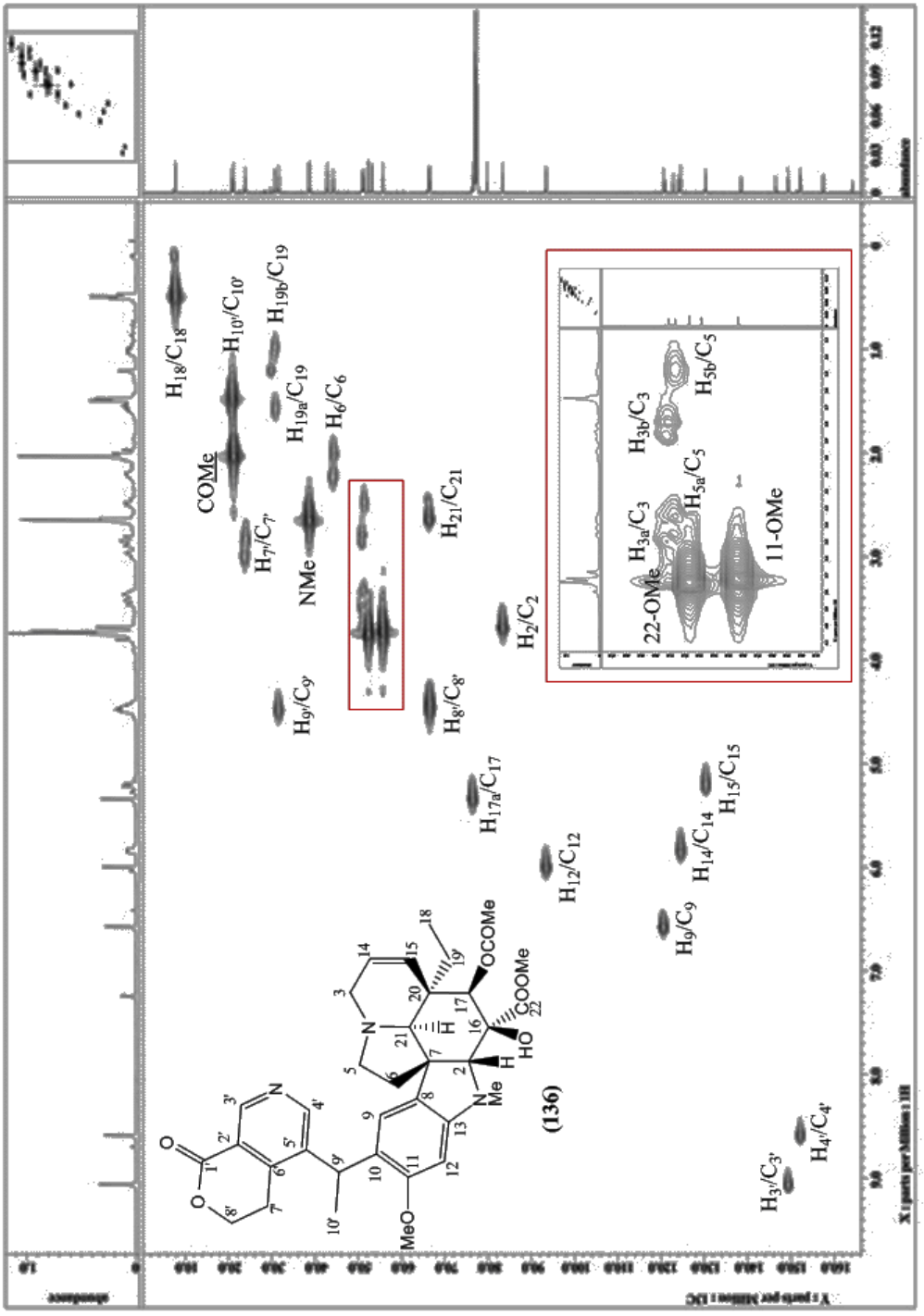


Figure 3.37: HMQC-NMR spectrum of alkaloid VII

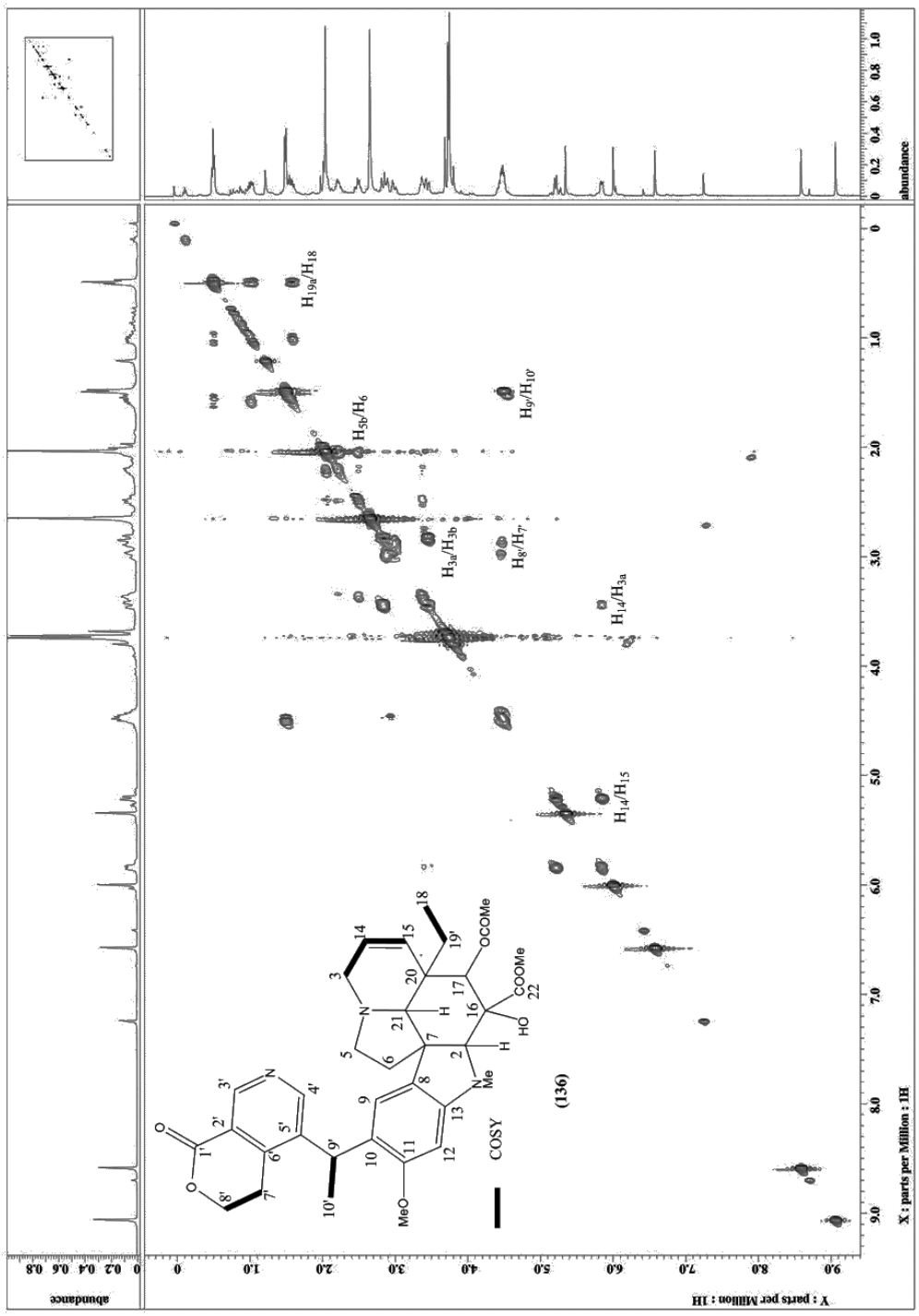


Figure 3.38: COSY-NMR spectrum of alkaloid VII

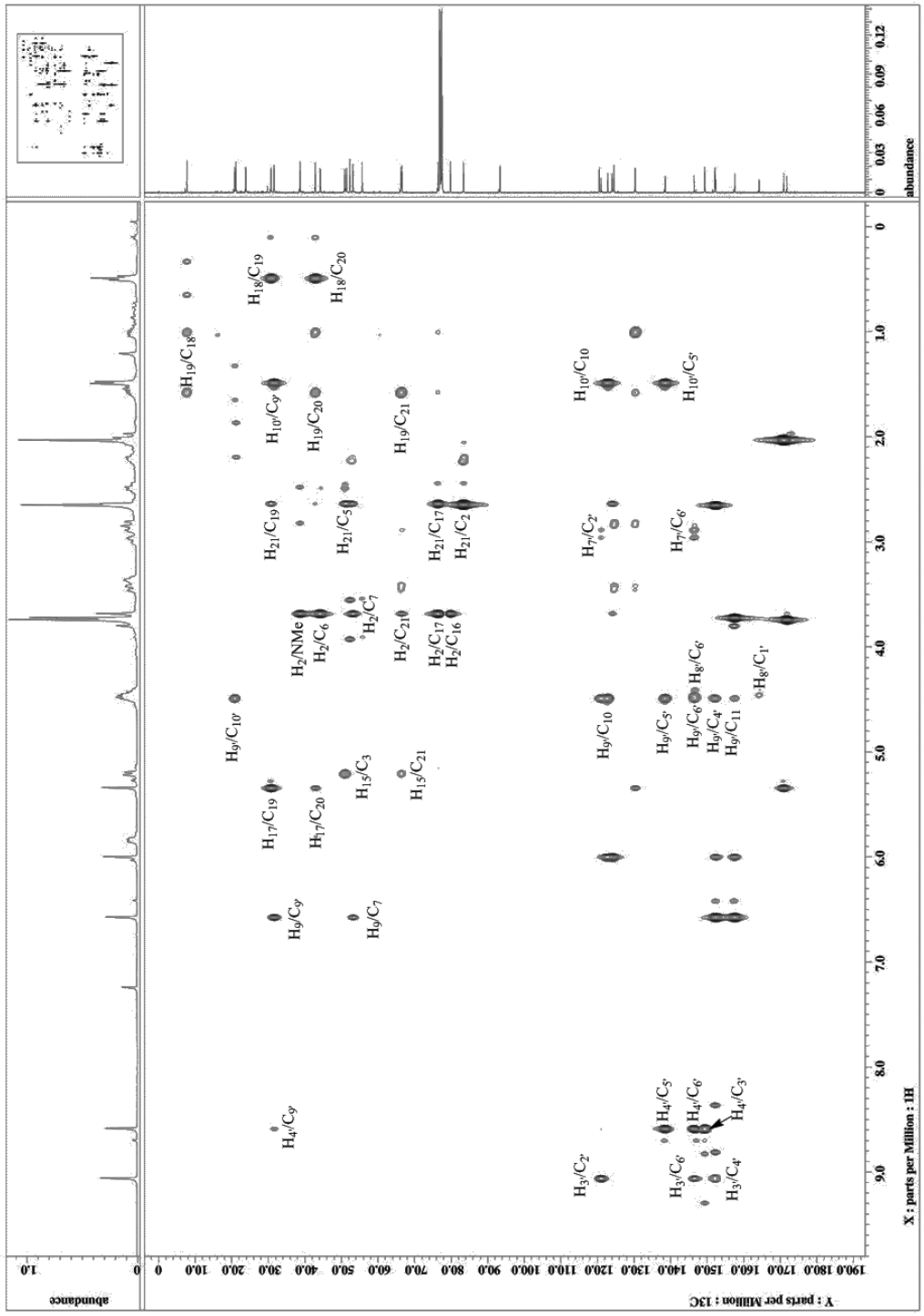


Figure 3.39: ^{1}H-^{13}C HMBC-NMR spectrum of alkaloid VII

CHAPTER 4

4.1 Bioactivity screening

Type I diabetes develops through autoimmune destruction of islet β-cells while dysregulation of insulin secretion from β-cells leads to type II diabetes.[114] Islet has poor deoxyribonucleic acid (DNA) repair capacity against oxidative damage and β-cells are vulnerable to oxidative stress. In diabetic individuals, there are imbalance of reactive oxygen species (ROS) generation and neutralization. Oxidative stress that damage cells plays a central role in the development of diabetic complication, insulin resistance and β-cells dysfunction.[115]

The present study was undertaken to scientifically investigate antidiabetic potential of *Catharanthus roseus'* dichloromethane extract (DE) and the isolated alkaloids using *in vitro* mouse pancreatic β-TC6 cells. Firstly, the β-TC6 cells were evaluated for cytotoxicity and cell proliferation together with antioxidant potential employing oxygen radical absorbance capacity (ORAC) assay. Furthermore, the DE and alkaloids effects on *in vitro* glucose uptake in β-TC6 cells using 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-2-yl)amino]-2-deoxyglucose (2-NBDG) were tested. Lastly, the mechanism of hypoglycemic activity that exhibited by selected alkaloids were evaluated through inhibition of the negative regulator of insulin signalling pathway, PTP-1B.

β-TC6 cells are derived from transgenic SV40 mice. It is an insulinoma cell lines that produced and secreted insulin in response to glucose stimulus. This cell line exhibited a 1.6-fold increase in insulin secretion in response to glucose stimulus, with a half-maximal response at 0.5 mM glucose. This cell line was also found to secrete glucagon and somatostatin.[114] Glucagon is a peptide hormone secreted by the pancreas, raises blood glucose levels.[116] Somatostatin is a peptide hormone that regulates the endocrine system and affects neurotransmission and cell proliferation.[117]

Glucose uptake ability evaluation in cells plays a fundamental role in diabetes mellitus research because there is a failure to increase glucose uptake into peripheral tissues in response to insulin in diabetes patient.[118] 2-NBDG is a fluorescent derivative of glucose that showed intense fluorescence at 542 nm when excited at 467 nm.[119] PTP-1B is a negative regulator of the insulin signaling pathway in human and is considered a promising potential therapeutic target for treatment of type 2 diabetes. For example, PTP-1B knock-out mice showed hyper obesity and proned to the development of type-2 diabetes.[120-122]

Colorimetric assays using the tetrazolium salt thiazolyl blue, also termed MTT (methyl-thiazolyl-tetrazolium) are widely used for assessment of cytotoxicity and proliferation studies in cell biology.[123-126] MTT gives a yellowish aqueous solution which will be reduces by dehydrogenases and reducing agents present in metabolically active cells to yield a water insoluble violet-blue formazan that can be estimated by spectrophotometer at 595 nm. The amount of MTT formazan is directly proportional to the number of living cells.[126]

ORAC is an integrative method for estimation of antioxidant capacity mainly for foods and beverages in United State of America.[127] In this study, antioxidant capacity was evaluated from the area under the curve (AUC) of the kinetic profiles of fluorescein. AUC values are compared with Trolox, a hydrosoluble vitamin E analog. Thus, allowing determination of ORAC index in terms of this reference compound. In ORAC assay, secondary reactions or even reactions associated with repair mechanism can be present.[128] Antioxidant-metal reaction could result in a lower concentration of antioxidants and therefore to an under-estimation of ORAC value.[129]

4.2 Effect of alkaloid on β-TC6 cell viability

The alkaloids were evaluated for cytotoxic activity on β-TC6 cells. The results were summarized in Table 4.1. The cells were treated for 24 hours with various concentrations of extracts and alkaloids. Cell viability was determined by MTT assays.

IC_{50} of extracts (HE, DE, ME & WA), vindoline **(4)**, vindorosine **(65)**, vindolicine **(90)** and serpentine **(104)** vindogentianine **(136)** exceeded 50 µg/mL, while, vindolinine **(9)** showed lowest IC_{50} at 20.5 ± 3.6 µg/mL followed by perivine **(45)** at 46.7 ± 4.4 µg/mL. On the other hand, doxorubicin, the standard drug exhibited IC_{50} at 3.8 ± 1.7 µg/mL. The present study demonstrated for the first time that these extracts, vindoline **(4)**, vindorosine **(65)**, vindolicine **(90)**, serpentine **(104)** and vindogentianine **(136)** are safe at high dosage, but vindolinine **(9)** and perivine **(45)** exhibited moderate cytotoxic effect against β-TC6 cells.

Table 4.1: Cell viability and ORAC in β-TC6 cells with treatment of extracts and alkaloid I-VII.

Extract/alkaloid	Cell viability IC_{50}		ORAC
	µg/ml	µM	TE ± SD µM
HE	68.0	-	134.0 ± 7.1
DE	78.4	-	66.5 ± 12.0
ME	165.2	-	18.0 ± 21.2
WA	232.4	-	87.0 ± 41.0
Vindoline **(4)**	82.1 ± 9.8	180.1 ± 21.5	41.0 ± 12.7
Vindolinine **(9)**	20.5 ± 3.6	57.6 ± 10.7	61.0 ± 19.8
Perivine **(45)**	46.7 ± 4.4	138.2 ± 13.0	29.0 ± 11.3
Vindorosine **(65)**	76.7 ± 8.1	180.1 ± 19.0	92.0 ± 11.3
Vindolicine **(90)**	68.0 ± 10.4	73.5 ± 11.3	185.5 ± 4.9
Serpentine **(104)**	62.5 ± 5.3	179.6 ± 15.2	24.0 ± 21.2
Vindogentianine **(136)**	110.7 ± 7.5	175.4 ± 11.9	64.5 ± 13.4

4.3 Oxygen radical absorbance capacity (ORAC) evaluation

The ORAC results were expressed as Trolox equivalent (TE). Net area under the curve (AUC) with increasing dosage of Trolox demonstrated a linearity correlation

with R^2 value 0.9668. (Figure 4.1) Quercetin, the positive control demonstrated highest TE value at 406.5 ± 2.1 µM. Out of the four extract tested for ORAC, only HE and DE showed significant activity at (134.0 ± 7.1 µM) and (66.5 ± 12.0 µM) respectively. Of all the isolated pure alkaloids, vindolicine (**90,** 185.5 ± 4.9 µM) demonstrated the highest antioxidant property followed by vindorosine (**65**, 92.0 ± 11.3 µM), vindogentianine (**136**, 64.5 ± 13.4 µM), vindolinine (**9**, 61.0 ± 19.8 µM)**,** vindoline (**4**, 41.0 ± 12.7 µM), perivine (**45**, 29.0 ± 11.3 µM) and serpentine (**104**, 24.0 ± 21.2 µM). The activity exhibited in DE extract may be due to the extract contain high abundance of vindoline **(4),** and minute quantity of vindolinine **(9)**, vindorosine **(65)**, vindolicine **(90)**, vindogentianine **(139)**.

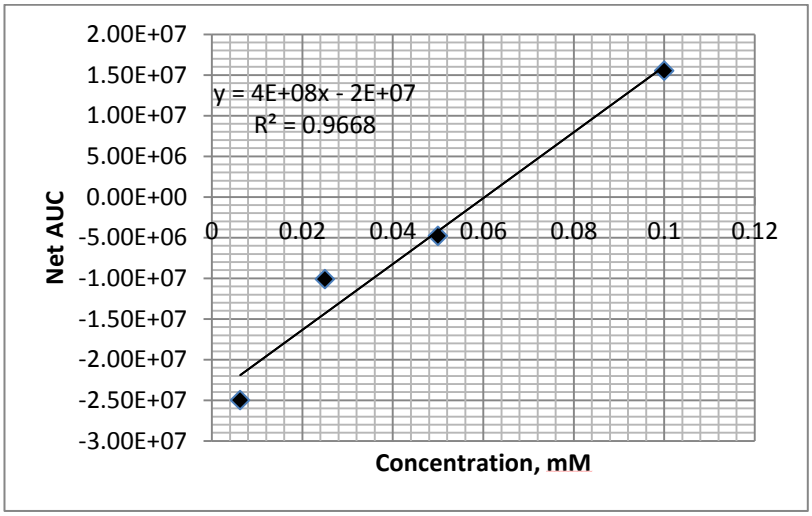


Figure 4.1: Net AUC with increasing dosage of Trolox correlation.

4.4 Effect of alkaloid on glucose uptake in β-TC6 cells

Improving glucose uptake in pancreatic cells could improve hyperglycemia condition of type 2 diabetes. The fluorescent glucose 2-NBDG could be visualized around cytosol in β-TC6 cells treated with DE and the alkaloids: vindoline **(4)**, vindolinine **(9)**, vindorosine **(65)**, vindolicine **(90)** and vindogentiamnine **(136)**. (Figure 4.3)

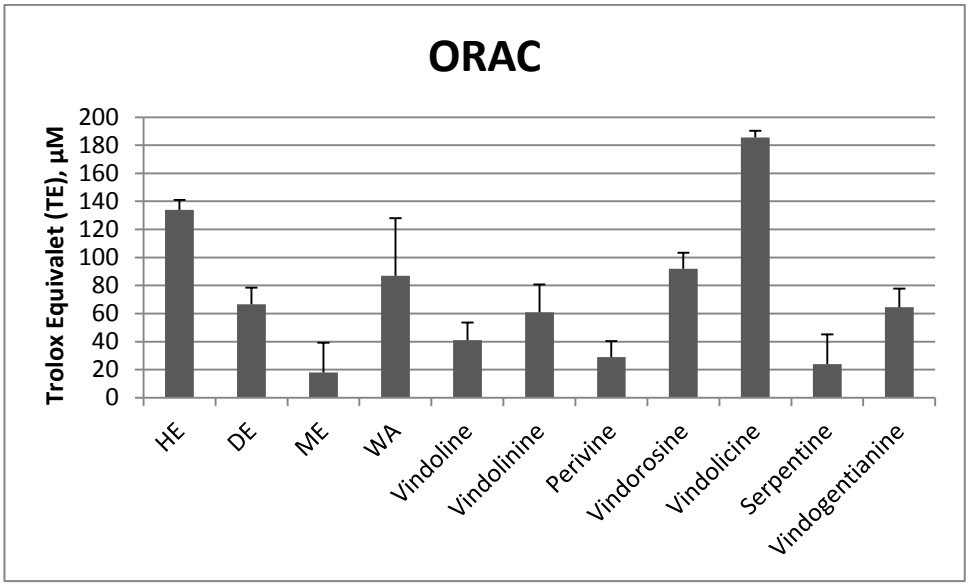


Figure 4.2: ORAC activity of extracts and alkaloids I-VII isolated from *Catharanthus roseus* leaves. Quercetin is included as positive control. (405 ± 2.1 µM)

DE dose-dependently increased glucose uptake, albeit the activity was relatively lower compare to the positive control, insulin (Figure 4.4). Among the 5 pure alkaloids, vindolicine **(90)** stimulated the highest glucose uptake in beta cells with more than 3 fold enhancement in glucose uptake compared to untreated control at 12.5 µg/mL (Figure 4.4). Glucose clearance rates in vindoline **(4)**, vindolinine **(9)**, vindorosine **(65)** and vindogentianine **(136)** treated β-TC6 cells were significantly higher after treatment (Figure 4.4). The weakest enhancement in glucose uptake was in beta cells treated with vindogentianine **(136)** with only 90 % increase glucose uptake was observed as compared to untreated beta cells at of 12.5 µg/mL (Figure 4.4).

At higher dosage (50.0 µg/mL), vindolinine **(9)** was cytotoxic towards β-TC6 cells (Figure 4.4). At the highest dosage (100.0 µg/mL), all five alkaloids exhibited cytotoxicity with large deviation on the reading of fluorescence intensity obtained (Figure 4.4). The results of glucose uptake showed in Figure 4.4 have $p<0.05$, except vindolinine at 50.0 µg/mL and all the treatment at 100.0 µg/mL.

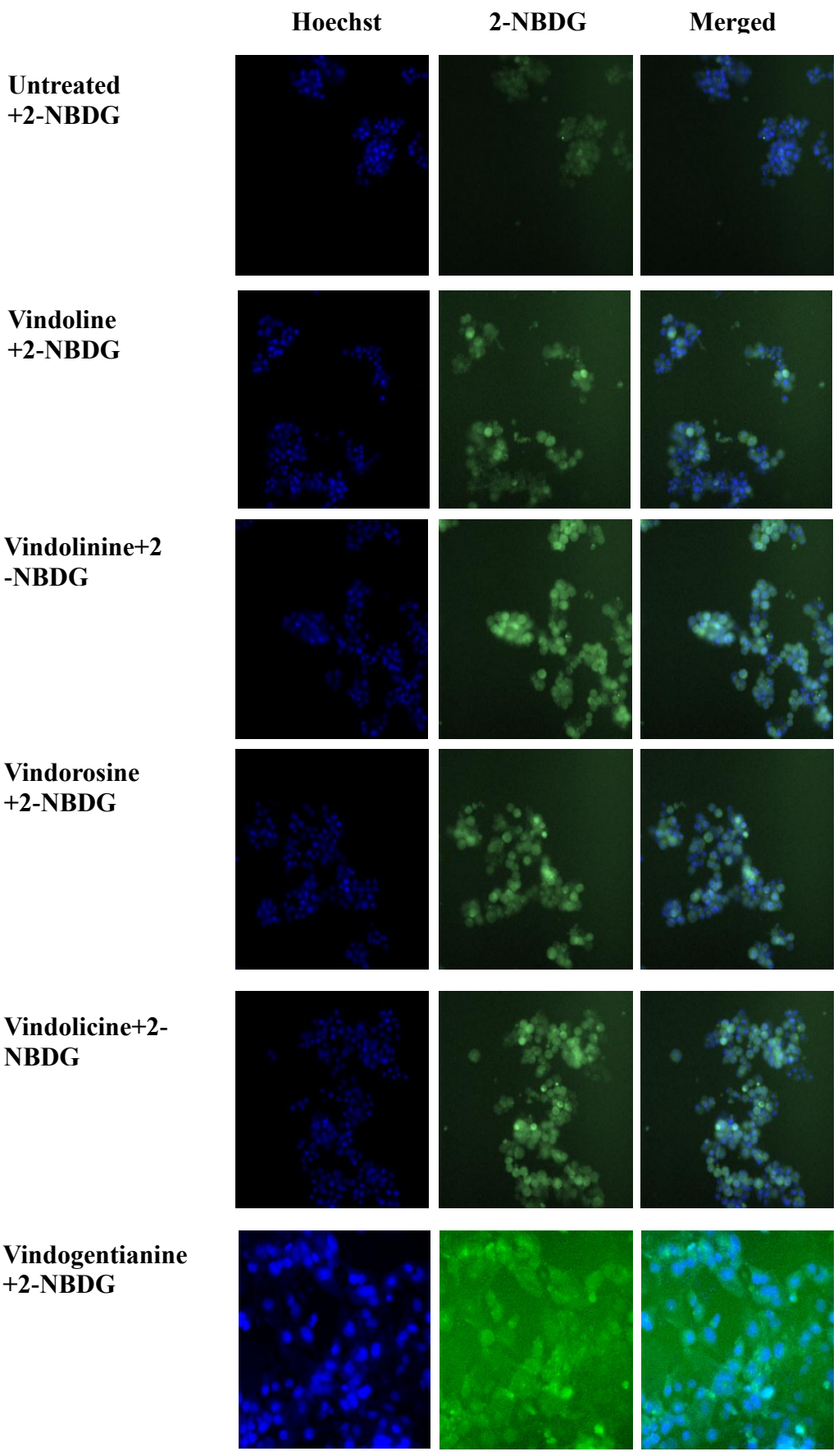


Figure 4.3**:** Representative photo showing enhanced glucose uptake by β-TC6 after treated with 25 µg/mL of selected alkaloids. Blue:Hoechst, Green: 2-NBDG

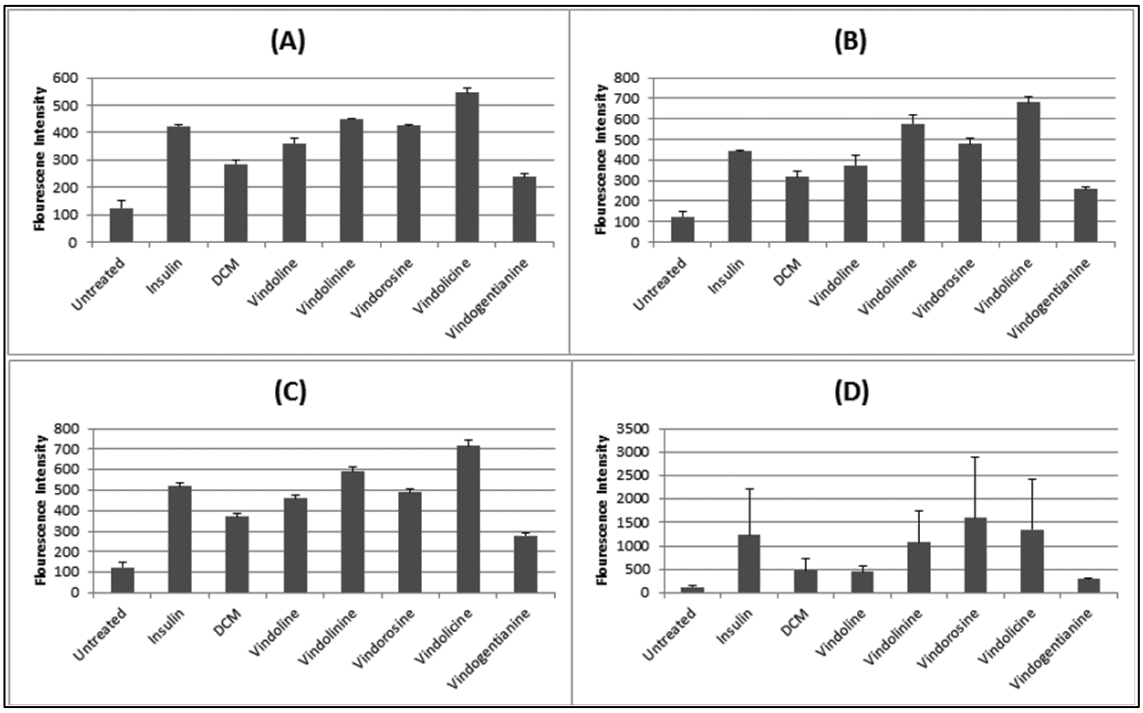


Figure 4.4: Bar chart showing fluorescent intensity of 2-NBDG taken up by β-TC6 cells. Insulin was included as positive controls. (A) 12.5 μg/mL, (B) 25.0 μg/mL, (C) 50.0 μg/mL and (D) 100.0 μg/mL

4.5 Effect of alkaloid on PTP-1B inhibition

The development of novel pharmaceutical agents that help ameliorate insulin resistance will be potentially important for the prevention and treatment of diabetes. PTP-1B is an enzyme that belongs to the Protein Tyrosine Phosphatase (PTP) family and a negative regulator of the insulin signaling pathway. To further evaluate antidiabetic potential of these compounds, we performed *in vitro* PTP-1B inhibition assays to determine whether four of the alkaloids, vindoline **(4)**, vindolinine **(9)**, vindorosine **(65)** and vindolicine **(90)** that exhibit highest activity in glucose uptake were active inhibitors of PTP-1B. Vindolicine **(90)** showed the highest inhibition activity follow by vindolinine **(9)**, vindorosine **(65)** and vindoline **(4)**. However, the activity of vindolicine **(90)** was relatively weaker as compared to the positive controls; RK-682 and ursolic acid (Figure 4.5).

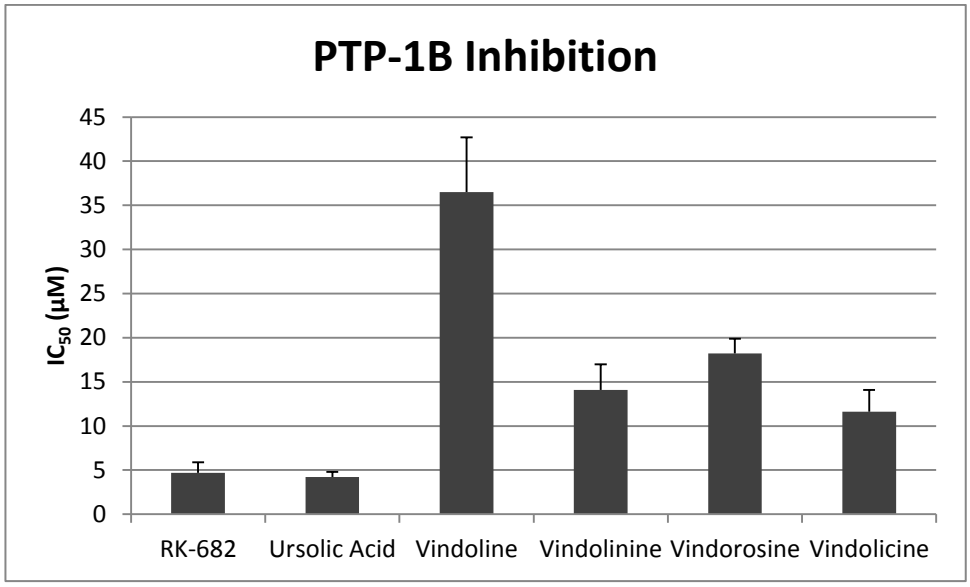


Figure 4.5: PTP-1B inhibition of selected alkaloids compared against positive control drugs RK-682 and Ursolic acid.

4.6 Discussion

In this study, the cytotoxicity and antioxidant potential of all the extracts were evaluated first by employing oxygen radical absorbance capacity (ORAC) assay in β-TC6 cells (normal pancreatic cells). The result of cytotoxicity and ORAC of DE directed for more detailed study of their alkaloids constituents because it showed significant Trolox equivalent (TE) value at 66.5 ± 12.0 µM as compared to the other extract that showed high standard deviation in this assay except HE. However, HE was not chosen to undergo alkaloid constituent study because it showed higher cytotoxicity against β-TC6 cells.

DE exhibited antioxidant and antidiabetic activity in β-TC6 mouse pancreatic cells. Moreover, it showed less cytotoxicity effect towards β-TC6 cells at high concentration. Based on these findings, there is a need to study the constituents from the active extract to discover the compound/s responsible for the antioxidant and hypoglycemic properties.We performed ORAC assay to assess the antioxidant property

of the alkaloids. Vindolicine **(90)** indicated the highest antioxidant property, followed by vindorosine **(65)** in ORAC values.

Out of the seven alkaloids successfully isolated from the DE, only plumeran alkaloids that include vindoline **(4)**, vindolinine **(9)**, vindorosine **(65)**, vindolicine **(90)** and vindogentianine **(136)**, were subjected to antidiabetic study by glucose uptake assay. Corynanthean alkaloids; Perivine **(45)** and serpentine **(104)** were not selected to undergo these studies because they showed weak ORAC with insignificant TE value at 29.0 ± 11.3 and 24.0 ± 21.2 µM respectively. Meanwhile, only the four best alkaloids in glucose uptake were subjected to PTP-1B inhibition evaluation.

In present study, the antidiabetic activity of vindorosine **(65)**, vindolicine **(90)** and vindogentianine **(136)** have been shown for the first time. The five alkaloids tested showed enhanced glucose uptake with dose dependent activity as compared with untreated β-TC6 cells. The lower dosage could provide antioxidant effect to β-TC6 cells, which promoted cellular activity, and led to the increase of glucose uptake. Cytotoxic effect (MTT IC_{50} = 20.5 ± 3.6 µg/mL) was observed in higher concentration of 50.0 µg/mL for vindolinine **(9)** and the other alkaloids at 100.0 µg/mL. Vindolicine **(90)** showed the greatest enhancement in glucose uptake. At the lowest dosage (12.5 µg/mL), the degree of glucose uptake increase in the order of vindogentianine **(136)**, vindoline **(4)**, vindorosine **(65)**, vindolinine **(9)** and vindolicine **(90)**, correlating with its PTP-1B inhibition activity, indicating that PTP-1B signaling could play a role in controlling cellular glucose uptake activity in β-TC6 cells.

To the best of our knowledge, this is the first report on PTP-1B inhibition study on all the four alkaloids; vindoline **(4)**, vindolinine **(9)**, vindorosine **(65)** and vindolicine **(90)**. These findings indicated that vindolicine **(90)** possessed the highest inhibition activity compared to the other three alkaloids.

CHAPTER 5

5.1 Conclusion

The chemical constituents study on the leaves of *Catharanthus roseus* yielded six known indole alkaloids which are vindoline **(4)**, vindolinine **(9)**, perivine **(45)**, vindorosine **(65)**, vindolicine **(90)**, serpentine **(104)** and a new indole alkaloids, vindogentianine **(136)**. Structural elucidations were established through spectroscopic methods: NMR, MS, UV, IR, optical rotation, elemental analysis and comparison with literature values.

Vindogentianine **(136)** is formed from the condensation of vindoline **(4)** and gentianine **(137)**. The latter is an artefact from reaction of ammonia with gentiopicroside **(138)**. Gentianine **(138)** has never occurred in *Catharanthus* but it has been reported in several Apocynanceae plants.

This study was also the first to show the cell viability of *Catharanthus roseus* extracts (HE, DE, ME, WA) and alkaloids from the leaves of *Catharanthus roseus* in normal mouse pancreatic cells, β-TC6 cells. The IC_{50} of extract decreases in the sequence of WA, ME, DE and HE with the most cytotoxic (IC_{50} = 68.0 μg/ml). Therefore, the consumption of water decoction from the leaves of *Catharanthus roseus* for diabetes treatment in traditional medicine practice considered safe, even though *Catharanthus roseus* contain many cytotoxic compounds. Evaluation of alkaloids isolated showed that most alkaloids were weakly cytotoxic except for vindolinine **(9)** and vindolicine **(90)** that showed moderate cytotoxicity against β-TC6 cells with IC_{50} of 57.6 ± 10.7 μM and 73.5 ± 11.3 μM respectively.

Antioxidant potential of this plant evaluated through ORAC showed that this plant possessed highest potential in HE and followed by DE. However, ME and WA did not show reliable data for comparison in ORAC. Most plumeran alkaloids showed

relatively better antioxidant potential in ORAC as compared to corynanthean alkaloids such as perivine **(45)** and serpentine **(104)**. Vindolicine **(90)** showed the highest antioxidant potential while the other alkaloids showed moderate or weak activity as compared to the positive control, quercetin.

The glucose uptake assay in β-TC6 cells with treatment of DE supported the antidiabetic activity of this plant as known from some earlier report and traditional medicinal usage. Vindorosine **(65)**, vindolicine **(90)** and vindogentianine **(136)** were shown to possess hypoglycemic activity for the first time. It showed increased glucose uptake with fluorescence intensity of 425.0 ± 1.4, 546.5 ± 16.3 and 237.0 ± 11.0 for vindorosine **(65)**, vindolicine **(90)** and vindogentianine **(136)** at concentration of 12.5 μg/mL compared with untreated β-TC6 cells (125.0 ± 25.0). Among all the alkaloids tested for glucose uptake, vindolicine **(90)** was found to be the most hypoglycemic. PTP-1B inhibition was shown to be one of the mechanisms underlying the hypoglycemic observed.

Therefore, vindolicine **(90)** is more beneficial and useful, relative to vindoline **(4)** or vindolinine **(9)** or vindorosine **(65)**, for amelioration of type 2 diabetes due to its' high antioxidant and PTP-1B inhibition activities. Vindolicine **(90)** being the most hypoglycemic and with the highest antioxidant activity is a good candidate to be investigated further as the source of new natural antidiabetic drug.

At the end, this plant unveils more interesting chemical constituents with isolation of alkaloid with evidence of gentianine **(137)** biogenetic occurrence in this plant regardless of how extensively had this plant been studied. Furthermore, it was found that many of these known alkaloids from this plant are antidiabetic. The water extract showed the highest antidiabetic activity suggesting more study should be conduct into it in future.

CHAPTER 6

6.1 Experimental

UV spectra were obtained in methanol using a Shidmazu UV-1650 PC UV-Vis spectrophotometer at concentration of 1 mg/mL. IR spectra were obtained on a Perkin-Elmer RX1 FT-IR spectrophotometer using potassium bromide pellets.

Column chromatography was performed on silica gel 60 (0.063-0.200 or 0.040-0.063 or 0.015-0.040 mm, MERCK) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB). Fractions were monitored by TLC (silica gel 60 F_{254} of MERCK) and spots were visualized under UV light (Spectroline Model ENF-260C/FE, 250V, 50 Hz and 0.17 AMPS) at wavelength of 254 nm and 365 nm followed by Dragendorff's reagent. Preparative TLC silica gel 60 F_{254} (250 μm) of MERCK was used in purification. All solvents except those used for bulky extraction (distilled) were AR grade.

6.2 Plant material

Catharanthus roseus (L.) G. Don. was cultivated at Jeli, Kelantan, Malaysia from November 2008 under natural condition. The leaves were collected around May 2009 and dried at 40 °C. The specimen was authenticated by Mr. Teo Leong Eng, a botanist in the Faculty of Science, University of Malaya. A voucher specimen with Herbarium No. of KL 5763 was deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

6.3 Extraction and fractionation

The dried leaves of *Catharanthus roseus* (1 kg) were sequentially extracted with n-hexane, dichloromethane and methanol. Firstly, dried grounded leaves of *Catharanthus roseus* (1 kg) was macerated with n-hexane (Hex) for 3 days at room temperature and was repeated twice. After removal of solvent, the plant residue was

first wetted with 25% ammonia for an hour followed by soaking with dichloromethane (DCM) by repeating the previous steps described for hexane extraction. After percolated with DCM, methanol (MeOH) was used as the final solvent to achieve universal extraction of the remaining metabolites in the leaves. Another 1 kg of the dried leaves of *Catharanthus roseus* were extracted with water under reflux for 3 hours. Water extract was obtained after freeze drying. As a result, we are able to obtain hexane (HE, 65.6 g), dichloromethane (DE, 44.5 g), methanol (ME, 252.4 g) and water (WA, 285.3 g) extract of *Catharanthus roseus* leaves (Table 6.1).

DE was used for studying the alkaloid constituents in the *Catharanthus rosues* leaves. DE (42.6 g) was subjected for acid-base extraction using 5% hydrochloric acid and 25% ammonia solution to obtain 4.2 g of crude alkaloid (DA) (Figure 6.1). DA was subjected to column chromatography (CC) with silica gel 60 (Merck, Germany) using gradient elution from DCM (100%) and DCM-MeOH (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, v/v). The CC was later flushed using DCM-MeOH (40:60, 20:80, v/v) and MeOH (100%). Each different solvent system, 1000 mL were used for elution. The eluent was collected in fractions of 100 mL. Those fractions with the same TLC profile were then combined. A total of 15 fractions were obtained (Fractions 1-15).

Table 6.1: Weight and percentage yield of different extraction solvent.

Extract	Weight (g)	Percentage Yield (%)
HE	65.5	6.55
DE	44.5	4.45
ME	252.4	25.24
WA	285.3	28.53

6.4 Isolation and purification

Fraction 3 (855.4 mg) was subjected to preparative TLC (PTLC) separation using Hex-EA-Acetone (20:78:2, v/v/v) under ammonia vapour on TLC silica gel 60

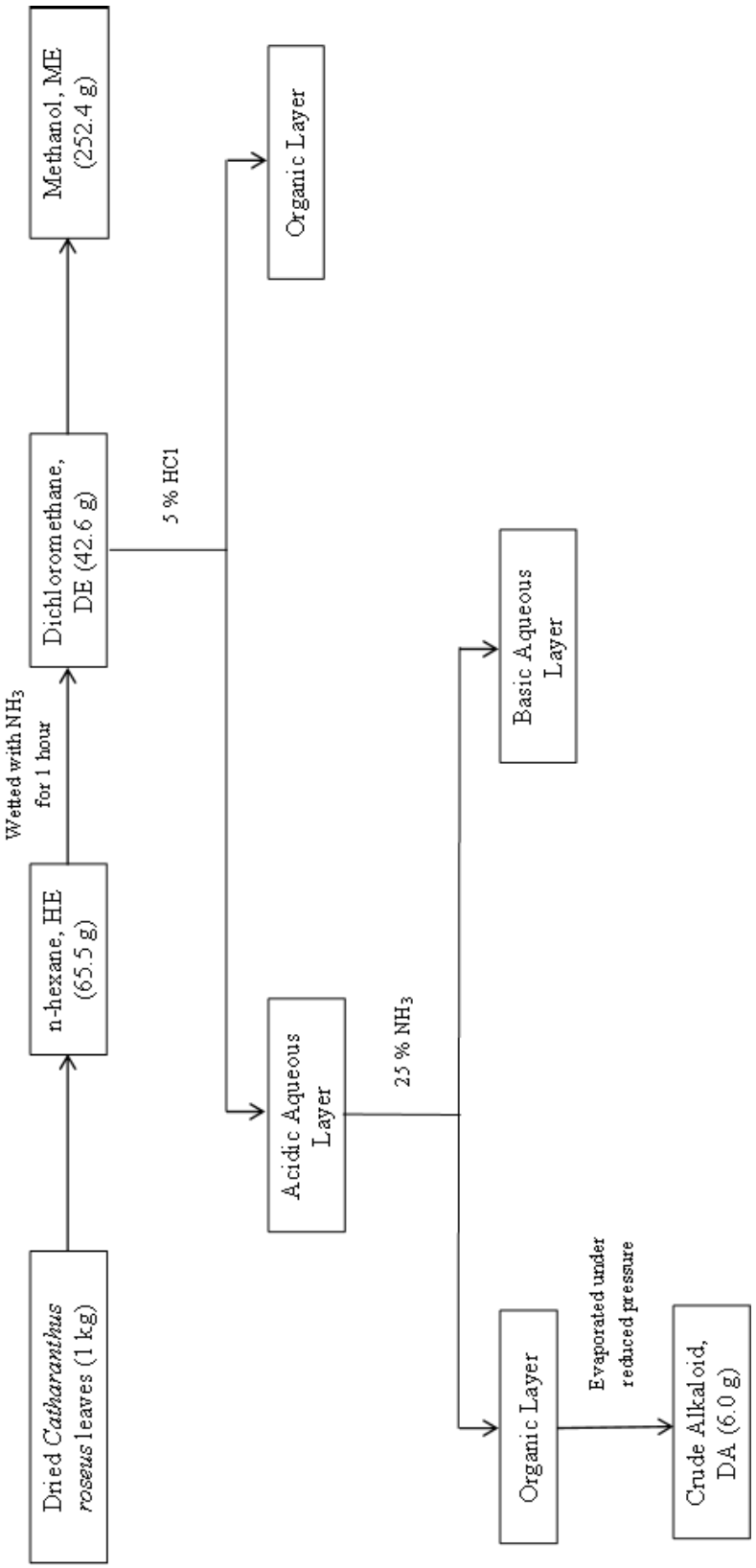


Figure 6.1: Solvent and acid-base extraction employed on the leaves of *Catharanthus rosues*.

F_{204} (Merck Germany) to yield alkaloid I: vindoline (**4**, 428.1 mg) and alkaloid IV: vindorosine (**65**, 57.9 mg). Fraction 4 (171.3 mg) was applied to PTLC with Hex-EA-Acetone (20:79:1, v/v/v) under ammonia vapour to yield alkaloid VII: vindogentianine (**136**, 38.6 mg) and alkaloid V: vindolicine (**90**, 31.0 mg). PTLC of fraction 6 (79.1 mg) using the same solvent system as applied on fraction 4 yielded alkaloid II: vindolinine (**9**, 8.4 mg) (Table 6.2 & Figure 6.2).

Fraction 9 (435.7 mg) was subjected to silica gel 60 (Merck, Germany) CC using gradient elution of the following composition: Hex:EA (70:30, 65:35, 60:40, 55:45, 50:50, 45:55, v/v) and EA (100%). Each 100 mL of different solvent system with 5 mL of TEA was used for elution. The eluent was collected in fractions of 10 mL. Sub-fractions with the same TLC profile were then combined based on ^{1}H NMR profiles to yield 9 sub-fractions. Sub-fraction 2 was subjected to PTLC using Hex-EA (60:40, v/v) with 5 mL of TEA to yield alkaloid III: perivine (**45**, 7.9 mg) (Table 6.2 & Figure 6.2).

Fraction 12 (67.8 mg) was chromatographed on sephadex LH-20 (GE Healthcare, Sweden) CC using MeOH-DCM (90:10, v/v, 160 mL) for elution. The eluent was collected in fractions of 10 mL to yield 14 sub-fractions. Sub-fractions 11-14 gave alkaloid VI: serpentine (**104**, 5.6 mg) (Table 6.2 & Figure 6.2).

Table 6.2: Chromatographic fractionation solvent and their respective alkaloids isolated with yield.

Solvent ratio DCM:MeOH	Fraction	Alkaloid	Weight (mg)	Yield (%)
80:20	3	I: Vindoline **(4)**	428.1	1.005
		IV: Vindorosine **(65)**	57.9	0.136
75:25	4	VII: Vindogentianine **(136)**	38.6	0.091
		V: Vindolicine **(90)**	31.0	0.073
65:35	6	II: Vindolinine **(9)**	8.4	0.020
55:45	9	III: Perivine **(45)**	7.9	0.019
50:50	12	VI: Serpentine **(104)**	5.6	0.013

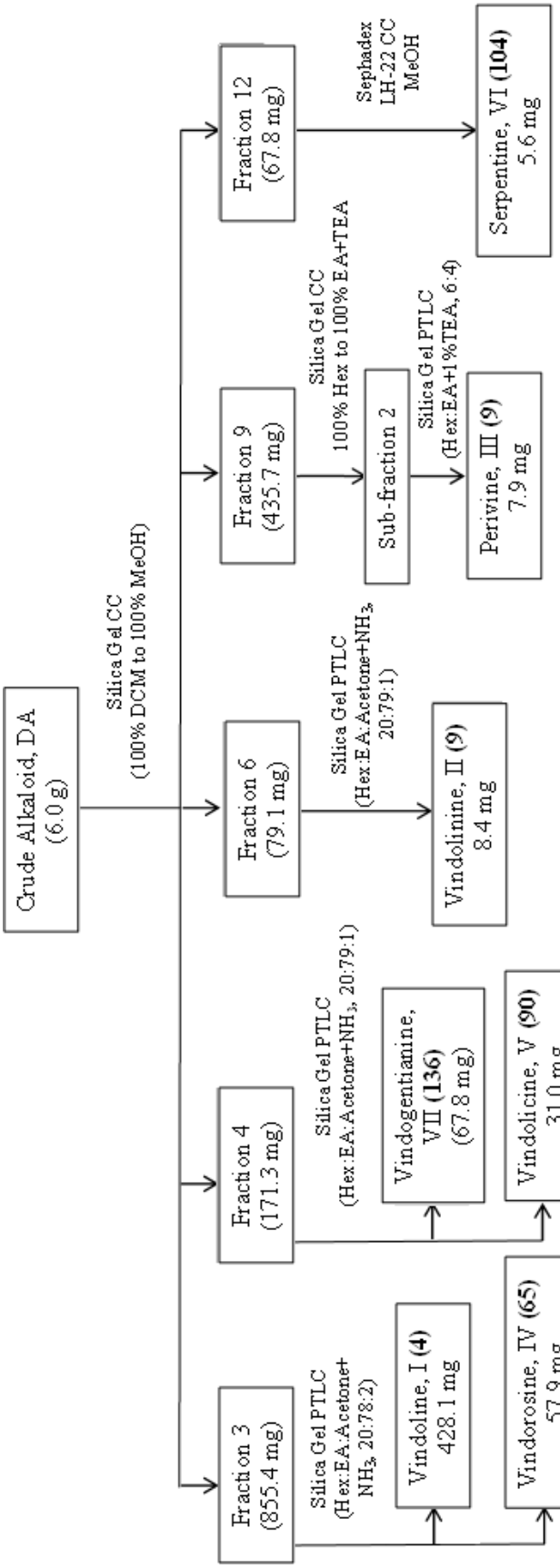


Figure 6.2: Chromatographic diagram of alkaloid I-VII from DA

6.5 Identification and characterization of alkaloids

One-dimensional and two-dimensional of ^{1}H and ^{13}C NMR experiments were carried out on a JEOL ECA 400 FT-NMR for vindoline (2), vindolinine (7), perivine (43), vindorosine (63), vindolicine (90), vindogentianine (139) and Bruker Ultrashield 600 plus FT-NMR with smartprobe for serpentine (130). $CDCl_3$ was used as the solvent for vindoline (2), vindolinine (7), vindorosine (63), vindolicine (90) and vindogentianine (139). Meanwhile, CD_3OD was the solvent for perivine (43) and serpentine (130). The chemical shift (δ) were recorded with reference to that of solvent, $CDCl_3$ (7.24 ppm) or CD_3OD (3.78 ppm) and the coupling constants were given in Hertz (Hz).

LC-MS for identification was carried out on Shimadzu LCMS-IT-TOF equipped with pump (LC-20AD), autosampler (SIL-20AC), column oven (CTO-20AC), PDA detector (SPD-M20A) and coupled to ESI interface (Shimadzu, Kyoto, Japan). Isolated compounds (5 μL) were injected and chromatographed on Waters XBridge C18 2.5 mm (2.1 x 50 mm) column with the mobile phase of A (0.1% formic acid in water) and B (0.1 % formic acid in MeOH). The mobile phase was performed in a step gradient as follows: isocratic 10% B for 2 min, 10% B to 100% B over the next 25 min and isocratic at 100% B for 5 min. The system was re-equilibrated for 5 min before the next run. Eluent were monitored using DAD at 220 nm, 254 nm, 350 nm and 450 nm. ESI-MS was performed in the range 100-2000 in both the positive and negative mode. The temperature of the heat block and curved desolvation line (the inlet for the high vacuum region) were set to 200°C and 250°C, respectively. Nitrogen gas was used as nebulizer with the flow rate set at 1.5 L/min. The ESI source voltage was set at 4.5 kV for positive mode and -3.5 kV for the negative mode whereas the detector was maintained at 1.7 kV. Shimadzu's LCMS solution software was used for data analysis.

6.6 Cell culture

Mouse β-TC6 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in 15% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). The cultures were kept in a humidified incubator at 37ºC in 5% CO_2 and the growth medium was changed every 3 days.

6.7 Cellular viability

The β-TC6 cells were used to determine the effect of extracts and alkaloids on cell growth by MTT assay.[134] Briefly, 1.5×10^4 β-TC6 cells were seeded into a 96-well plate and incubated at 37°C in 5% CO_2 for 24 h. The second day, the seeded cells were treated with alkaloids and extracts. After another 24h of incubation, MTT solution was added at 2 mg/mL and the plates were kept in incubator at 37°C in 5% CO_2 for 1 h. Absorbance at 570 nm was measured by a Plate Chameleon V microplate reader (Hidex, Turku, Finland). The obtained values are expressed as a percentage of cell viability after exposure to extracts and alkaloids for 24h. Cell viability was defined as the ratio (percentage) of the absorbance of treated cells to that of untreated ones.

6.8 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was carried out based on the procedure described by Cao *et al.* (1993) with slight modifications.[130] In brief, the sample/blank (175 μL) was dissolved in PBS at the concentration of 160 μg/mL at pH 7.4. The standard Trolox was prepared in serial dilutions starting from 75 mM. Standard 96-well black microplates was used for the assay, and 25 μL each of the samples (extract and alkaloids), standard (Trolox), blank (solvent/PBS), or positive control (quercetin) were added to the wells. Fluorescent sodium salt solution was added at 150 μL per well, followed by incubation at 37°C for 45 min. The total volume of each well was made up to 200 μL by adding 2,20-azobis

(2-amidinopropane) dihydrochloride (AAPH) solution. Fluorescence value was recorded at 37°C until it became 0 (excitation at 485 nm, emission at 535 nm) using a fluorescence spectrophotometer (Perkin-Elmer LS 55) equipped with an automatic thermostatic autocell-holder. Data were collected every 2 min for 2 h and the data analysis was subsequently done by calculating the differences of areas under the fluorescein decay curve (AUC) between the blank and the sample. The resulted values were expressed as Trolox equivalents.

6.9 2-NBDG glucose uptake

The cells were seeded at a density of 1.5×10^4 cells/mL in a 96-well plate and allowed to attach, spread, and proliferate to near confluence at 37°C in 5% CO_2. After overnight incubation, the medium was discarded, washed with phosphate-buffered saline (PBS) twice and replenished with 2.5 mM glucose in basal medium comprising DMEM without glucose or pyruvate supplemented with L-glutamine and 15% (v/v) FBS (final serum glucose concentration of approximately 0.25 mM). The cells were incubated for 60 min at 37°C in 5% CO_2. The conditioning medium was then replaced with 10 mM 2-NBDG (Invitrogen) in basal medium in the presence or absence of DE and alkaloids. The cells were kept at 37°C in 5% CO_2 for 30 min to permit endocytosis of the 2-NBDG. Next, the medium was removed, cells were washed twice with PBS and stained with nucleic dye Hoechst 33342 for another 30 min. The cells were then observed for intra-cellular fluorescence at *Excitation*/*Emission* = 350 nm/461 nm and *Excitation*/*Emission* = 475 nm/550 nm for Hoechst 33342 and 2-NBDG, respectively. Plates were evaluated using the ArrayScan High Content Screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA) and analyzed with Target Activation BioApplication software (Cellomics Inc.).[131]

6.10 PTP-1B inhibition

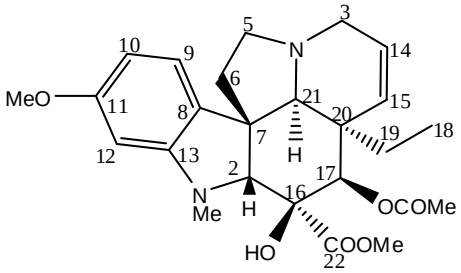
The pNPP (*para*-Nitro Phenyl Phosphate) was used as substrate to assay the phosphatase activity.[132] The ionic strength of the assay buffer (pH 7.4), was adjusted to 0.15 M using NaCl. The buffer contains 50 mM 3,3-dimethyl glutarate, 1 mM EDTA, 5 mM glutathione, and 0.5% FCS (not heat inactivated). Briefly, diluted (50 μM alkaloids) and undiluted inhibitors were added to the reaction mixture containing 0 or 2.5 mM pNPP to reach the total volume of 100 μL. The enzyme (recombinant PTP-1B) was added to initiate the reaction. The reaction was allowed to proceed for 5 min before adding the inhibitor. The incubation was continued at 37°C for 5-60 min and the time was recorded. Then 20 μL 0.5 M NaOH in 50% ethanol was added to stop the reaction. To determine the enzyme activity, the absorbance was measured at 405 nm using Tecan ELISA reader with appropriate corrections for absorbance of substrate, alkaloids, and nonenzymatic hydrolysis of substrate.

6.11 Statistical analysis

Data were presented as means ± SEM and analyzed with unpaired Student's *t*-test in which $p<0.05$ is considered significant.

6.12 General spectral data of the isolated alkaloids

Vindoline (4)[97-101]



(4)

Nature of isolate : yellowish brown, amorphous solid

Molecular formula : $C_{25}H_{32}N_2O_6$

Optical rotation : $[\propto]_D^{22}$ -46° (*c* 1.0, MeOH)

UV (MeOH) λ_{max} : 224 (5.00), 251 (4.79) and 307 (4.63) nm

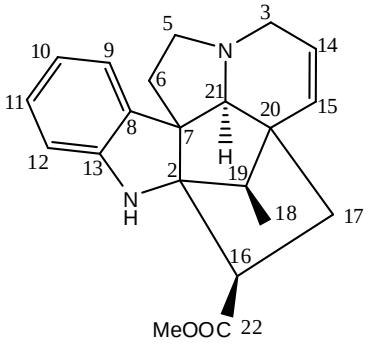
IR (KBr) υ_{max} : 3458, 1741 and 1240 cm^{-1}

$[M+H]^+$: 457 *m/z*

^{1}H-NMR : 400 MHz; $CDCl_3$; Me_4Si (Refer Table 3.1)

^{13}C-NMR : 100 MHz; $CDCl_3$; Me_4Si (Refer Table 3.1)

Vindolinine (9)[97-98;102]



(9)

Nature of isolate : brown, amorphous solid

Molecular formula : $C_{21}H_{24}N_2O_4$

Optical rotation : $[\propto]_D^{22}$ -46° (*c* 1.0, MeOH)

UV (MeOH) λ_{max} : 3365, 1725, 1466 and 1246 cm^{-1}

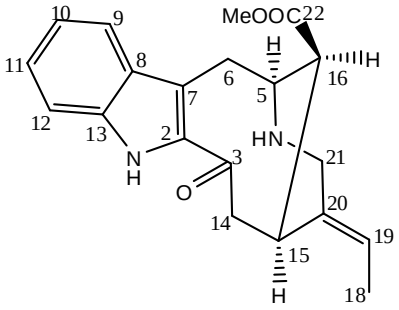
IR (KBr) υ_{max} : 216 (4.14), 244 (3.95), 295 (3.77) and 308 (3.71) nm

$[M+H]^+$: 337 *m/z*

^{1}H-NMR : 400 MHz; $CDCl_3$; Me_4Si (Refer Table 3.2)

^{13}C-NMR : 100 MHz; $CDCl_3$; Me_4Si (Refer Table 3.2)

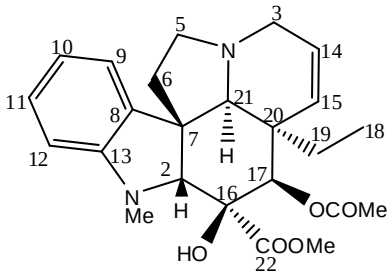
Perivine (45)[97;103]



(45)

Nature of isolate	: brown, amorphous solid
Molecular formula	: $C_{20}H_{22}N_2O_3$
Optical rotation	: $[\propto]_D^{22}$ -76° (*c* 1.0, MeOH)
UV (MeOH) λ_{max}	: 228 (4.36), 296 (4.11) and 314 (4.29) nm
IR (KBr) υ_{max}	: 3311, 1725 and 1643 cm^{-1}
$[M+H]^+$	: 339 *m/z*
^{1}H-NMR	: 400 MHz; CD_3OD; Me_4Si (Refer Table 3.3)
^{13}C-NMR	: 100 MHz; CD_3OD; Me_4Si (Refer Table 3.3)

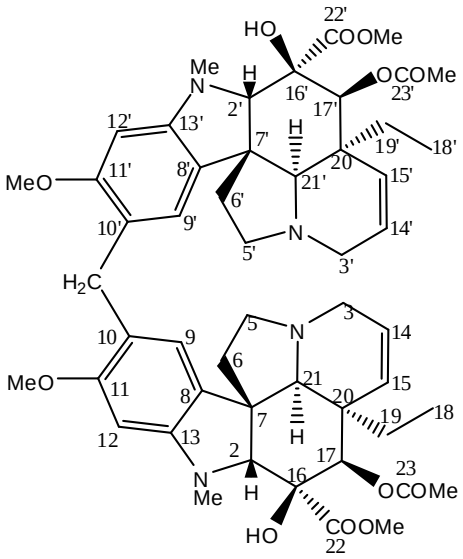
Vindorosine (65)[97-100]



(65)

Nature of isolate	: brown, amorphous solid
Molecular formula	: $C_{24}H_{30}N_2O_5$
UV (MeOH) λ_{max}	: 203, 252 and 308 nm
IR (KBr) υ_{max}	: 203 (4.16), 252 (3.83) and 308 (3.50) nm
$[M+H]^+$	: 427 *m/z*
^{1}H-NMR	: 400 MHz; $CDCl_3$; Me_4Si (Refer Table 3.4)
^{13}C-NMR	: 100 MHz; $CDCl_3$; Me_4Si (Refer Table 3.4)

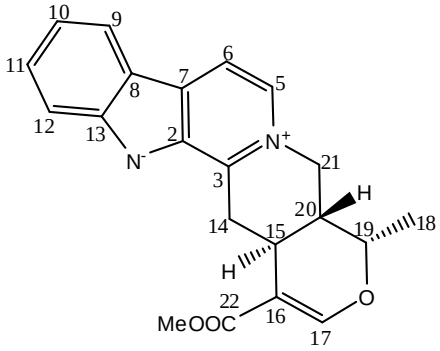
Vindolicine (90)[71;97-98]



(90)

Nature of isolate	: brown, amorphous solid
Molecular formula	: $C_{51}H_{64}N_4O_{12}$
Optical rotation	: $[\propto]_D^{22}$ -75° (*c* 1.0, MeOH)
UV (MeOH) λ_{max}	: 217 (4.61), 253 (4.37) and 308 (4.03) nm
IR (KBr) υ_{max}	: 3432, 1741 and 1241 cm^{-1}
$[M+H]^+$	: 925 *m/z*
^{1}H-NMR	: 400 MHz; $CDCl_3$; Me_4Si (Refer Table 3.5)
^{13}C-NMR	: 100 MHz; $CDCl_3$; Me_4Si (Refer Table 3.5)

Serpentine (104)[104]



(104)

Nature of isolate : brown, amorphous solid

Molecular formula : $C_{21}H_{20}N_2O_3$

Optical rotation : $[\propto]_D^{22}$ +60° (*c* 1.0, MeOH)

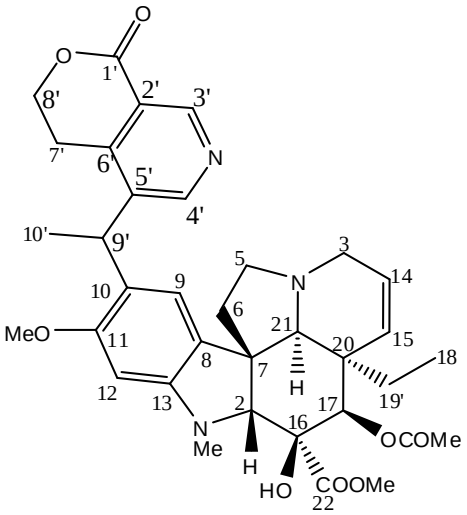
UV (MeOH) λ_{max} : 251 (4.20), 308 (4.02) and 363 (3.58) nm

$[M+H]^+$: 349 *m/z*

^{1}H-NMR : 600 MHz; CD_3OD; Me_4Si (Refer Table 3.6)

^{13}C-NMR : 150 MHz; CD_3OD; Me_4Si (Refer Table 3.6)

Vindogentianine (136)[97-101;105]



(136)

Nature of isolate : brown, amorphous solid

Molecular formula : $C_{35}H_{41}N_3O_8$

Optical rotation : $[\propto]_D^{22}$ -134° (*c* 1.0, MeOH)

UV (MeOH) λ_{max} : 216 (4.46), 259 (4.11) and 310 (4.01) nm

IR (KBr) υ_{max} : 3458, 1741 and 1240 cm^{-1}

$[M+H]^+$: 632 *m/z*

^{1}H-NMR : 400 MHz; $CDCl_3$; Me_4Si (Refer Table 3.7)

^{13}C-NMR : 100 MHz; $CDCl_3$; Me_4Si (Refer Table 3.7)

REFERENCES

1 Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine, 27*(1), 1-93.

2 Balunas, M.J., Kinghorn, A.D. (2005). Drug discovery from medicinal plants. *Life Sciences, 78*(5), 431-441.

3 Newman, D.J., Cragg, G.M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Product, 75*, 311-335.

4 Newman, D.J., & Cragg, G.M. (2007). Natural Product as sources of New Drugs over the Last 25 Years. *Journal of Natural Product*, 461-477.

5 Grover, J.K., Yadav, S., Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology, 81*, 81-100.

6 Li, W.L., Zheng, H.C, Bukuru, J., De Kimpe, N. (2004). Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. *Journal of Ethnopharmacology, 92*(1), 1-21.

7 Duan, X., Mao, S. (2010). New strategies to improve the intranasal absorption of insulin. *Drug Discovery Today*, *15*(11–12), 416-427.

8 Hossain, P., Kawar, B., & El Nahas, M. (2007). Obesity and diabetes in the developing world - a growing challenge. *New England Journal of Medicine, 356*(3), 213-215.

9 National Diabetes Data Group. (1979). Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes*, *28*(12), 1039-1057.

10 Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes estimates for the year 2000 and projections for 2030. *Diabetes care*, *27*(5), 1047-1053.

11 Letchuman, G.R., Wan Nazaimoon, W.M., Wan Mohamad, W.B., Chandran, L.R., Tee, G.H., Jamaiyah, H., Isa, M.R., Zanariah, H., Fatanah, I., Ahmad Faudzi, Y. (2010). Prevalence of Diabetes in the Malaysian National Health Morbidity Survey III 2006. *Medical Journal of Malaysia, 65*(3), 173-179.

12 Aslam, J., Khan, S.H., Siddiqui, Z.H., Fatima, Z., Maqsood, M., Bhat, M.A. (2010). *Catharanthus roseus* (L.) G. Don. An Important Drug: It's Applications and Production. *Pharmacie Globale (International Journal of Comprehensive Pharmacy), 4*(12), 1-16.

13 van Bergen, M.A., Snoeijer, W. (1996). *Revision of Catharanthus G. Don. Series of Revisions of Apocynaceae XLI*. Netherlands: Backhuys Publishers.

14 Pradhan, S. (2011). Antihyperglycemic effect of various medicinal plants of Sikkim Himalayas - a review. *International Journal of Research in Phytochemistry & Pharmacology, 1*, 124-130.

15 van de Venter, M., Roux, S., Bungu, L.C., Louw, J., Crouch, N.R., Grace, O.M., Maharaj, V., Pillay, P., Sewnarian, P., Bhagwandin, N., Folb, P. (2008). Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. *Journal of Ethnopharmacoologyl, 119,* 81-86.

16 Kevin, L.Y.W., Hussin, A.H., Zhari, I., Chin, J.H. (2012). Sub-acute oral toxicity study of methanol leaves extract of *Catharanthus roseus* in rats. *Journal of Acute Disease*, 38-41.

17 Ong, H.C., Ahmad, N., Milow, P. (2011). Traditional Medicinal Plants Used by the Temuan Villagers in Kampung Tering, Negeri Sembilan, Malaysia. *Ethno Med, 5*(3), 169-173.

18 Nammi, S., Boini, M.K., Lodagala, S.D., Behara, R.B. (2003). The juice of fresh leaves of *Catharanthus roseus* Linn. reduces blood glucose in normal and alloxan diabetic rabbits. *BMC Complementary and Alternative Medicine, 3*(4), 2.

19 Singh, S.F., Vats, P., Suri, S., Shyam, R., Kumria, M.M, Ranganathan, S., Sridharan, K. (2001). Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology, 76*(3), 269-277.

20 Marles, R.J., Farnsworth, N.R. (1995). Antidiabetic plants and their active constituents. *Phytomedicine, 2*(2), 137-189.

21 Ganga, R.M., Satyanarayana, S., Eswar, K.K. (2012). Safety of Gliclazide with the aqueous extract of *Vinca rosea* on pharmacodynamic activity in normal and alloxan induced diabetic rats. *Journal of Pharmacy Research, 5*, 1555-1558.

22 Svoboda, G.A. (1969). The alkaloids of *Catharanthus roseus* G. Donn (*Vinca rosea* L.) in Cancer Chemotherapy. In Gunkel, J.E. (Ed.), *Current topics in plant science.* (pp. 303-335). New York: Academic Press.

23 Gacche, R.N., Dhole, N.A. (2011). Profile of aldose reductase inhibition, anti-cataract and free radical scavenging activity of selected medicinal plants: An attempt to standardize the botanicals for amelioration of diabetes complications. *Food and Chemical Toxicology., 49,* 1806-1813.

24 Ohadoma, S.C., Michael, H.U. (2011). Effects of co-administration of methanol leaf extract of *Catharanthus roseus* on the hypoglycemic activity of metformin and glibenclamide in rats. *Asian Pacific Journal of Tropical Medicine, 4*(6), 475-477.

25 Hickey, M., King, C. (1988). *100 Families of Flowering Plants* (pp. 346-351). Cambridge: Cambridge University Press.

26 Siddiqui, M.J., Ismail, Z., Aisha, A.F.A., Abdul Majid, A.M.S. . (2010). Cytotoxic Activity of *Catharanthus roseus* (Apocynaceae) Crude Extracts and Pure Compounds against Human Colorectal Carcinoma Cell Line. *International Journal of Pharmacology, 6*(1), 43-47.

27 Warren, W. (1996). *Tropical Flowers of Malaysia & Singapore*. Singapore: Periplus Editions.

28 Marlinza, R. (2012). Effect of Pasak Bumi (Eurycoma longifolia Jack) Root In Precopulation Stage to the Fertility of Female Mouse (Mus musculus L.). *Biospecies*, *2*(2).

29 Noble, R.L., Beer, C.T., & Cutts, J.H. (1958). Role of chance observations in chemotherapy: Vinca rosea. *Ann N Y Acad Sci, 76*(3), 882-894.

30 Kuboyama, T., Yokoshima, S., Tokuyama, H., Fukuyama, T. (2004). Stereocontrolled Total Synthesis of (+)-Vincristine. *PNAS, 101*(33), 11966-11970.

31 Ferreres, F., Pereira, D.M., Valentão, P., Andrade, P.B., Seabra, R.M., Sottomayor, M. (2008). New Phenolic Compounds and Antioxidant Potential of *Catharanthus roseus*. *Journal of Agricultural and Food Chemistry, 56*, 9967-9974.

32 Verpoorte, R., & Mustafa, N.R. (2007). Phenolic compounds in *Catharanthus roseus. Phytochemistry Reviews, 6*, 243-258.

33 Toki, K., Saito, N., Irie, Y., Tatsuzawa, F., Shigihara, A., & Honda, T. (2008). 7-O-Methylated anthocyanidin glycosides from *Catharanthus roseus*. *Phytochemistry, 69*(5), 1215-1219.

34 Chung, I.M., Ali, Chun, S.C., San, K.Y., Peebles, C.A.M. & Ahmad, A. (2008). New Catharanthusopimaranoside A and B from Hairy Root Cultures of *Catharanthus roseus*. *Chemistry of Natural Compounds, 44*(4), 458-462.

35 Guedes De Pinho, P., Gonçalves, R.F., Valentão, P., Pereira, D.M., Seabra, R.M., Andrade, P.B., & Sottomayor. M. (2009). Volatile composition of *Catharanthus roseus* (L.) G. Don using solid-phase microextraction and gas chromatography/mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 49*, 674-685.

36 Leeuwenberg, A.J. (1980). The Taxonomic Position of Some Genera in Longaniaceae, Apocynaceae and Rubiaceae, related families which Contain Indole Alkaloids. In Phillipson, J.D., Zenk, M.H. (Ed.), *Indole and Biogenetically Related Alkaloids* (pp. 1-10). New York: Academic Press.

37 Wang, L., Zhang, Y., He, H.P., Li, S.F., & Hao, X.J. (2011). Three New Terpenoid Indole Alkaloids from *Catharanthus roseus*. *Planta Medica, 77*, 754-758.

38 Gorman, M., & Neuss, N. (1964). The chemistry of some monomeric Catharanthus alkaloids. *Lloydia, 27*, 393-396.

39 Siddiqui, M.J.A., Ismail, Z., & Saidan, N.H. (2011). Simultaneous determination of secondary metabolites from *Vinca rosea* plant extractives by reverse phase high performance liquid chromatography. *Pharmacognosy Magazine, 7*, 92-96.

40 Lee, S.L., Reporter, M., Hsu, S.N., Corbin, J.L., Luli, G., Burkhouse, P.L., et al. (1988). The lipid composition and conversion of tryptamine to Nb-acetyltryptamine in a *Catharanthus roseus* cell line without indole alkaloids. *Journal of Industrial Microbiology and Biotechnology., 3*, 293-297.

41 Kutney, J.P., Choi, L.S.L., Kolodziejczyk, P., Sleigh, S.K., Stuart, K.L., Worth, B.R., et al. (1981). Alkaloid production in *Catharanthus roseus* cell cultures. V. Alkaloids from the 176G, 299Y, 340Y and 951G cell lines. *Journal of Natural Products, 44*, 536-540.

42 Mukhopadhyay, S., & Cordell, G.A. (1981). Catharanthus alkaloids. XXXVI. Isolation of vincaleukoblastine (VLB) and periformyline from *Catharanthus trichophyllus* and pericyclivine from *Catharanthus roseus*. *Journal of Natural Products, 44*, 335-339.

43 Atta ur, R., Hasan, S., & Qulbi, M.R. (1985). β-Carboline from *Catharanthus roseus*. *Planta Medica*, 287.

44 Gueritte, F., Langlois, N., & Petiard, V. (1983). Secondary metabolites isolated from *Catharanthus roseus* tissue culture. *Journal of Natural Products, 46*, 144-148.

45 Stoeckigt, J., & Soll, H.J. (1980). Indole alkaloids from cell suspension cultures of *Catharanthus roseus* and *C. ovalis*. *Planta Medica, 40*, 22-30.

46 Atta ur, R., & Bashir, M. (1983). Isolation of new alkaloids from *Catharanthus roseus*. *Planta Medica, 49*, 124-125.

47 Atta ur, R., Ali, I., Bashir, M., & Choudhary, M.I. (1984). Isolation and structure of rosamine - a new pseudoindoxyl alkaloid from *Catharanthus roseus*. *Zeitschrift für Naturforschung. Teil b, Anorganische Chemie, organische Chemie, 39(9)*, 1292-1293.

48 Vachnadze, V.Y., Malikov, V.M., Il'yasova, K.T., Mudzhiri, K.S., & Yunusov, S.Y. (1973). Qualitative characteristics of alkaloids of some species of Vinca-genera. *Khimiya Prirodnykh Soedinnenii*, (1), 72-76.

49 Habib ur, R., & Atta ur, R. (2005). NMR studies on alioline. A novel vinca alkaloid. *Zeitschrift für Naturforschung. Teil b, Anorganische Chemie, organische Chemie, 60*, 870-874.

50 Zhong, X., Wang, G., Wang, Y., Zhang, X., & Ye, W. (2010). Monomeric indole alkaloids from the aerial parts of *Catharanthus roseus*. *Yaoxue Xuebao, 45*, 471-474.

51 Atta ur, R., Ali, I., & Bashir, M. (1984). Isolation of rhazimol from the leaves of *Catharanthus roseus*. *Journal of Natural Products, 47*, 389.

52 Atta ur, R., Ali, I., & Bashir, M. (1984). Gomaline - a new indolenine alkaloid from *Catharanthus roseus*. *Heterocycles, 22*, 85-86.

53 Stolle, K., & Groeger, D. (1967). *Catharanthus roseus*-a new medicinal plant. *Pharm Zentralh Deut, 106,* 285-306.

54 Kohl, W., Witte, B., & Hoefle, G. (1982). Alkaloids from *Catharanthus roseus* tissue cultures. III. *Zeitschrift für Naturforschung. Teil b, Anorganische Chemie, organische Chemie, 37B,* 1346-1351.

55 Kohl, W., Witte, B., Sheldrick, W.S., & Hoefle, G. (1984). Indole alkaloids from *Catharanthus roseus* cell cultures. IV: 16R-19,20-E-isositsirikine, 16R-19,20-Z-isositsirikine and 21-hydroxycyclolochnerine. *Planta Medica, 50,* 242-244.

56 Nguyen, N.S., Nguyen, N.H., & Tran, H. (1984). Preliminary study of the chemical composition of *Catharanthus roseus*, Apocynaceae. *Tap Chi Hoa Hoc, 22,* 5-8.

57 Atta ur, R., Ali, I., & Chaudhary, M.I. (1986). Bannucine - a new dihydroindole alkaloid from *Catharanthus roseus* (L) G. Don. *Journal of Chemical Society, Perkin Transition 1,* 923-926.

58 Atta ur, R., Ali, I., & Chaudhry, M.I. (1985). Isolation and carbon-13 NMR studies on cathovaline, an alkaloid from the leaves of *Catharanthus roseus*. *Planta Medica,* 447-448.

59 Peraza-Sanchez, S.R., Gamboa-Angulo, M.M., Erosa-Lopez, C., Ramirez-Erosa, I., Escalante-Erosa, F., Pena-Rodriguez, L.M., et al. (1998). Production of 19(S)-epimisiline by hairy root cultures of *Catharanthus roseus*. *Natural Product Letters, 11,* 217-224.

60 Atta ur, R., Fatima, J., & Albert, K. (1984). Isolation and structure of rosicine from *Catharanthus roseus*. *Tetrahedron Letters, 25,* 6051-6054.

61 Kohl, W., Witte, B., & Hoefle, G. (1981). Alkaloids from *Catharanthus roseus* cell cultures. II. *Zeitschrift für Naturforschung. Teil b, Anorganische Chemie, organische Chemie, 36B,* 1153-1162.

62 Atta ur, R., Bashir, M., Kaleem, S., & Fatima, T. (1984). Isolation, structure and oxidative fragmentation of 16-epi-19-S-vindolinine - an alkaloid from the leaves of *Catharanthus roseus*. *Zeitschrift für Naturforschung. Teil b, Anorganische Chemie, organische Chemie, 39B,* 695-700.

63 Cuellar, A., & O'Farrill, T.H. (1976). Venalstonine: a new alkaloid isolated from the roots of *Catharanthus roseus* G. Don growing in Cuba. Revista *Cubana* de Farmacia, *10,* 3-18.

64 Svoboda, G.H., Gorman, M., & Tust, R.H. (1964). Alkaloids of *Vinca rosea*. XXV. Lochrovine, perimivine, vincoline, lochrovidine, lochrovicine, and vincolidine. *Lloydia, 27,* 203-213.

65 Zhou, H., Tai, Y., Sun, C., & Pan, Y. (2005). Rapid identification of Vinca alkaloids by direct-injection electrospray ionisation tandem mass spectrometry

and confirmation by high-performance liquid chromatography-mass spectrometry. *Phytochemical Analysis, 16*, 328-333.

66 El-Sayed, A., & Cordell, G.A. (1981). Catharanthus alkaloids. XXXIV. Catharanthamine, a new antitumor bisindole alkaloid from *Catharanthus roseus* (Apocynaceae). *Journal of Natural Products, 44*, 289-293.

67 Pyuskyulev, B.D., Tam, M.N., Danieli, B., Passarella, D., & Lesma, G. (1995). Phytochemical studies on the indole alkaloids of *Catharanthus roseus*, cultivated in Bulgaria. *Bulgarian Chemical Communications, 28*, 175-186.

68 De Bruyn, A., De, T.L., Simonds, R., Verzele, M., & De, P.C. (1982). Alkaloids from *Catharanthus roseus*. Isolation and identification of 17-desacetoxyvinblastine and 17-desacetoxyleurosine. *Bulletin des Sociétés Chimiques Belges, 91*, 75-85.

69 Atta ur, R., Alam, M., Ali, I., Habib ur, R., & Haq, I. (1988). Leurosinone. A new binary indole alkaloid from *Catharanthus roseus*. *Journal of Chemical Society, Perkin Transition 1*, 2175-2178.

70 El-Sayed, A., Handy, G.A., & Cordell, G.A. (1980). Catharanthus alkaloids. XXXIII. 21'-Oxo-leurosine from *Catharanthus roseus* (Apocynaceae). *Journal of Natural Products, 43*, 157-161.

71 El-Sayed, A., Handy, G.A., Cordell, G.A. (1983). Catharanthus alkaloids. XXXVIII. Confirming structural evidence and antineoplastic activity of the bisindole alkaloids leurosine-N'b-oxide (pleurosine), roseadine and vindolicine from *Catharanthus roseus*. *Journal of Natural Products, 46*, 517-527.

72 Mukhopadhyay, S., & Cordell, G.A. (1981). Catharanthus alkaloids. XXXV. Isolation of leurosidine N'b-oxide from *Catharanthus roseus*. *Journal of Natural Products, 44*, 611-613.

73 Tafur, S., Jones, W.E., Dorman, D.E., Logsdon, E.E., & Svoboda, G.H. (1975). Alkaloids of *Vinca rosea* (*Catharanthus roseus*). XXXVI. Characterization of new dimeric alkaloids. *Journal of Pharmaceutical Science, 64*, 1953-1957

74 Gueritte, F., Nguyen, V.B., Langlois, Y., & Potier, P. (1980). Biosynthesis of antitumor alkaloids from *Catharanthus roseus*. Conversion of 20'-deoxyleurosidine into vinblastine. *Journal of Chemical Society, Chemical Communications*, 452-453.

75 Dorman, D.E., & Paschal, J.W. (1976). Carbon-13 NMR spectroscopy. Comparison of the spectra of some dimeric Catharanthus alkaloids and their derivatives. *Organic Magnetic Resonance, 8*, 413-418.

76 Miller, J.C., Gutowski, G.E., Poore, G.A., & Boder, G.B. (1977). Alkaloids of *Vinca rosea* L. (*Catharanthus roseus* G. Don). 38. 4'-Dehydrated derivatives. *Journal of Medicinal Chemistry, 20*, 409-413.

77 Roepke, J., Salim, V., Wu, M., Thamm, A.M.K., Murata, J., Ploss, K., et al. (2010). Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. *PNAS, 107*, 15287-15292.

78 Tafur, S., Occolowitz, J.L., Elzey, T.K., Paschal, J.W., & Dorman, D.E. (1976). Alkaloids of *Vinca rosea*. (*Catharanthus roseus*). XXXVII. Structure of vincathicine. *Journal of Organic Chemistry, 41*, 1001-1005.

79 Jossang, A., Fodor, P., & Bodo, B. (1998). A New Structural Class of Bisindole Alkaloids from the Seeds of *Catharanthus roseus*: Vingramine and Methylvingramine. *Journal of Organic Chemistry, 63*, 7162-7167.

80 Battersby, A.R., Burnett, A.R., Hall, E.S., & Parsons, P.G. (1968). Rearrangement process in indole alkaloid biosynthesis. *Chemical Communications*, 1582-1583.

81 Petiard, V., Courtois, D., Gueritte, F., Langlois, N., & Mompon, B. (1982). New alkaloids in plant tissue cultures. In *Plant tissue culture 1982: proceedings, 5th International Congress of Plant Tissue and Cell Culture held at Tokyo and Lake Yamanake, Japan, July 11-16, 1982/edited by Akio Fujiwara*. Tokyo: Japanese Association for Plant Tissue Culture.

82 Prista, L.N., Ferreira, M.A., Roque, A.S., & Alves, A.C. (1964). Chromatographic studies of the alkaloids of *Vinca roseus*. II. Sample from Madeira. *Garcia de Orta, 12*, 295-308.

83 Stoeckigt, J., Husson, H.P., Kan-Fan, C., & Zenk, M.H. (1977). Cathenamine, a central intermediate in the cell free biosynthesis of ajmalicine and related indole alkaloids. *Journal of Chemical Society, Chemical Communications*, 164-166.

84 Balsevich, J., DeLuca, V., & Kurz, W.G.W. (1986). Altered alkaloid pattern in dark-grown seedlings of *Catharanthus roseus*. Isolation and characterization of 4-desacetoxyvindoline, a novel indole alkaloid and proposed precursor of vindoline. *Heterocycles, 24*, 2415-2421.

85 Scott, A.I., & Qureshi, A.A. (1969). Biogenesis of Strychnos, Aspidosperma, and Iboga alkaloids. Structure and reactions of preakuammicine. *Journal of American Chemical Society, 91*, 5874-5876.

86 Nobert, N. (1970). Indole Alkaloids. In Pelletier, S.W. (Ed.), *Chemistry of the Alkaloids* (pp. 213-266). New York: Van Nostrand Reinhold Company.

87 Swan, G.A. (1967). Introduction. In Swan, G.A. (Ed.), *An Introduction to the Alkaloids* (pp. 1-17). Oxford: Blackwell Scientific Publication.

88 Verpoorte, R., & Schripsema, J. (1994). Isolation, Identification, and Structure Elucidation of Alkaloids A General Overview. In Linkskens, H.F., & Jackson, J. F. (Ed.), *Modern Methods of Plant Analysis Volume 12 Alkaloids.* (pp. 1-24). Berlin: Springer-Verlag.

89 Kumar, B., & Chopra, H.K. (2005). *Biogenesis of Natural Products*. Harrow, Middlesex: Alpha Science International Ltd.

90 Mann, J., Davidson, R.S., Hobbs, J.B., Banthrope, D.V., & Harborne, J.B. (1994). *Natural products: their chemistry and biological significance*. Harlow, Essex: Longman.

91 Radwanski, E. R., & Last, R. L. (1995). Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. *The Plant Cell*, *7*(7), 921.

92 Contin, A., van der Heijden, R., Lefeber, A.W.M., & Verpoorte, R. (1998). The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. *FEBS Letters*, *434*(3), 413-416.

93 Takahashi, S., & Murata, N. (2006). Glycerate-3-phosphate, produced by CO fixation in the Calvin cycle, is critical for the synthesis of the D1 protein of photosystem II. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1757*(3), 198-205.

94 Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S., & Sahm, H. (1996). Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *Journal of the American Chemical Society*, *118*(11), 2564-2566.

95 O'Connor, S.E., & Maresh, J.J. (2006). Chemistry and biology of monoterpene indole alkaloid biosynthesis. *Natural Product Reports*, *23*(4), 532-547.

96 Herrmann, K. M., & Weaver, L. M. (1999). The shikimate pathway. *Annual review of plant biology*, *50*(1), 473-503.

97 Pavia, D.L., Lampman, G.M., Kriz, G.S. & Vyvyan, J.R. (2009). *Introduction to Spectroscopy Fourth Edition*. Belmont: Brooks/Cole Cencage Learning.

98 Gorman, M., Neuss, N., Svoboda, G.H., Barnes Jr, A.J. & Cone, N.J. (1959). A note on the alkaloids of *Vinca rosea* Linn (*Catharanthus roseus* G. Don.) II: catharanthine, lochnericine, vindolinine and vindoline. *Journal of the American Pharmaceutical Association*, *48*(4), 256-257.

99 Gorman, M., Neuss, N., Biemann, K. (1962). Vinca alkaloid X The structure of vindoline. *Journal of American Chemical Society*, *84*, 1058-1059.

100 Wenkert, E., Cochran, D.W., Hagaman, E.W., Schell, F.M., Neuss, N., Katner, A.S. (1973). Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Naturally Occuring Substances. XIX. Aspidorsperma Alkaloids. *Journal of American Chemical Society, 95*(15), 4990-4995.

101 Wang, C.H., Wang, G.C., Wang, Y., Zhang, X.Q., Huang, X.J., Zhang, D.M., Chen, M.F., Ye, W.C. (2012). Cytotoxic dimeric indola alkaloids from *Catharanthus roseus*. *Fitoterapia, 83,* 765-769.

102 Durham, L.J., Shoolery, J.N., Djerassi, C. (1974). Reinvestigation of the proton resonance spectrum of vindolinine at 300 MHz. *PNAS. , 71*(10), 3797-3799.

103 Pascale Clivio, B.R., Hadi, H.A., David, B., Sevenet, T., Zeches, M., & Le Man-Olivier, L. (1990). Alkaloids from Leaves and Stem Bark of *Ervatamia Polyneura*. *Phytochemistry, 29*(9), 3007-3011.

104 Wachsmuth, O., Matusch, R. (2002). Anhydrnium bases from *Rauvolfia serpetina*. *Phytochemistry 61*, 705-709.

105 Cordell, G.A. (1977). Chapter 8 The Monoterpene Alkaloids. In Manske, R.H.F. (Ed.), *The Alkaloids: Chemistry and Physiology* (Vol. 16, pp. 431-510). New York: Academic Press.

106 Coscia, C.J., Botta, L., & Guarnaccia, R. (1970). On the mechanism of iridoid and secoiridoid monoterpene biosynthesis. *Archives of Biochemistry and Biophysics, 136*(2), 498-506.

107 Krstić, D., Janković, T., Aljančić, I., Šavikin-Fodulović, K., Menković, N., & Milosavljević, S. (2004). Phytochemical investigation of *Gentiana dinarica*. *Biochemical Systematics and Ecology, 32*(10), 937-941.

108 Wang, D., Xu, M., Zhu, H.T., Chen, K.K., Zhang, Y.J., & Yang, C.R. (2007). Biotransformation of gentiopicroside by asexual mycelia of *Cordyceps sinensis*. *Bioorganic & Medicinal Chemistry Letters, 17*(11), 3195-3197.

109 Seigler, D.S. (1977). Chapter 1 Plant Systematics and Alkaloids. In Manske, R.H.F. (Ed.), *The Alkaloids: Chemistry and Physiology* (Vol. 16, pp. 1-82). New York: Academic Press.

110 Vercauteren, J., Massiot, G., Sévenet, T., Richard, B., Lobjois, V., Le Men-Olivier, L., et al. (1981). Alkaloids of *Alstonia lanceolata*. *Phytochemistry, 20*(6), 1411-1413.

111 Cherif, A., Massiot, G., Le Men-Olivier, L., Pusset, J., & Labarre, S. (1989). Alkaloids of *Alstonia coriacea*. *Phytochemistry, 28*(2), 667-670.

112 Legseir, B., Cherif, A., Richard, B., Pusset, J., Labarre, S., Massiot, G., et al. (1986). Alkaloids of *Alstonia lenormandii*, a structural revision of 10-methoxycompactinervine. *Phytochemistry, 25*(7), 1735-1738.

113 Lavaud, C., Massiot, G., Vercauteren, J., & Le Men-olivier, L. (1982). Alkaloids of *Hunteria zeylanica*. *Phytochemistry, 21*(2), 445-447.

114 Hohmeier, H.E., Newgard, C.B. (2004). Cell lines derived from pancreatic islets. *Molecular and Cellular Endocrinology, 228*(1–2), 121-128.

115 Bensellam, M., Laybutt, D.R., Jonas, J.C. (2012). The molecular mechanisms of pancreatic β-cell glucotoxicity: Recent findings and future research directions. *Molecular and Cellular Endocrinology, 364*(1–2), 1-27.

116 Kreymann, B., Ghatei, M. A., Williams, G., & Bloom, S. R. (1987). Glucagon-like peptide-1 7-36: a physiological incretin in man. *The Lancet, 330*(8571), 1300-1304.

117 Patel, Y. C. (1992). General aspects of the biology and function of somatostatin. In *somatostatin* (pp. 1-16). Springer Berlin Heidelberg.

118 Friedman, J. (2002). Fat in all the wrong places. *Nature, 415*, 268-269.

119 Yoshioka, K., Takahashi, T., Homma, T., Saito, M., Oh, K.B., Nemoto, Y., et al. (1996). A novel fluorescent derivative of glucose applicable to assesment of glucose uptake activity of *Escherichia coli*. *Biochimica et Biophysica Acta, 1289*, 5-9.

120 Goldstein, B.J. (2001). Protein-tyrosine phosphatase 1B (PTP1B): a novel therapeutic target for type 2 diabetes mellitus, obesity and related states of insulin resistance. *Current Drug Targets - Immune, Endocrine* & *Metabolic Disorders, 1*(3), 265-275.

121 Zhang, Z.Y., Lee, S.Y. (2003). PTP1B inhibitors as potential therapeutics in the treatment of type 2 diabetes and obesity. *Expert Opinion* on *Investigational Drugs, 12*(2), 223-233.

122 Montalibet, J., Skorey, K., McKay, D., Scapin, G., Asante-Appiah, E., Kennedy, B.P. (2006). Residues distant from the active site influence protein-tyrosine phosphatase 1B inhibitor binding. *Journal of Bioogical Chemistry, 281*(8), 5258-5266.

123 Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1–2), 55-63.

124 Horobin, R.W., Kiernan, J.A. (2002). *Conn's biological stains. A handbook of dyes, stains and flourochromes for use in biology and medicine* (10th ed.). Oxford: Bios Scientific Publishers.

125 Berridge, M.V., Herst, P.M., Tan, A.S. (2005). Tetrazolium dyes as tools in cell biology: new insight into their cellular reduction. *Biotechnology Annual Review, 11*, 127-152.

126 van Merloo, J., Kasper, G.J., Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Methods Molecular Biology, 731*, 237-245.

127 USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2. U.S. Department of Agriculture.

128 Bisby, R.H., Brooke, R., Navaratnam, S. (2008). Effect of antioxidant oxidation potential in the oxygen radical absorption capacity (ORAC) assay. *Food Chemistry, 108*(3), 1002-1007.

129 Nkhili, E., Brat, P. (2011). Reexamination of the ORAC assay: effect of metal ions. *Analytical and bioanalytical chemistry, 400*(5), 1451-1458.

130 Cao, G., Alessio, H.M., Cutler, R.G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine, 14*(3), 303-311.

131 Loaiza, A., Porras, O.H., Barros, L.F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *Journal of Neuroscience, 23*(19), 7337-7342.

132 Lund, I.K., Andersen, H.S., Iversen, L.F. (2004). Structure-based design of selective and potent inhibitors of protein-tyrosine phosphatase β. *The Journal of Biological Chemistry, 279*(23), 24226–24235.

PUBLICATION

Molecules **2013**, *18*, 9770-9784; doi:10.3390/molecules18089770

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Article

Antidiabetic and Antioxidant Properties of Alkaloids from *Catharanthus roseus* (L.) G. Don

Soon Huat Tiong [1], Chung Yeng Looi [2], Hazrina Hazni [1], Aditya Arya [2,3], Mohammadjavad Paydar [2], Won Fen Wong [4], Shiau-Chuen Cheah [5], Mohd Rais Mustafa [2] and Khalijah Awang [1,*]

[1] Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; E-Mails: kingdom86_@hotmail.com (S.H.T.); hazrinahazni@um.edu.my (H.H.)

[2] Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia; E-Mails: looicy@um.edu.my (C.Y.L.); aditya@um.edu.my (A.A.); paybarmj@gmail.com (M.P.); rais@um.edu.my (M.R.M.)

[3] Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

[4] Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia; E-Mail: wonfen@um.edu.my

[5] Faculty of Medicine and Health Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, Cheras 56000 Kuala Lumpur, Malaysia; E-Mail: cheahsc@ucsi.university.edu.my

* Author to whom correspondence should be addressed; E-Mail: khalijah@um.edu.my; Tel.: +603-796-740-64; Fax: +603-796-741-93.

Received: 17 June 2013; in revised form: 2 August 2013 / Accepted: 9 August 2013 / Published: 15 August 2013

Abstract: *Catharanthus roseus* (L.) G. Don is a herbal plant traditionally used by local populations in India, South Africa, China and Malaysia to treat diabetes. The present study reports the *in vitro* antioxidant and antidiabetic activities of the major alkaloids isolated from *Catharanthus roseus* (L.) G. Don leaves extract. Four alkaloids—vindoline **I**, vindolidine **II**, vindolicine **III** and vindolinine **IV**—were isolated and identified from the dichloromethane extract (DE) of this plant's leaves. DE and compounds **I**–**III** were not cytotoxic towards pancreatic β-TC6 cells at the highest dosage tested (25.0 μg/mL). All four alkaloids induced relatively high glucose uptake in pancreatic β-TC6 or myoblast C2C12 cells, with **III** showing the highest activity. In addition, compounds **II**–**IV** demonstrated good protein tyrosine phosphatase-1B (PTP-1B) inhibition activity, implying their therapeutic potential against type 2 diabetes. **III** showed the highest antioxidant